Understanding protein tyrosine phosphatase sigma function: dimer formation and interacting proteins

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A PhD thesis submitted in fulfilment of the requirements in the University of London

16 January 2007

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Abstract

Cytoplasmic and transmembrane protein tyrosine phosphatases (PTPs) provide the enzymatic counterbalance to protein tyrosine kinase activity. PTP sigma (PTPσ) is an adhesion molecule-like receptor PTP (RPTP) that is expressed on the growth cones of developing axons. PTPσ binds ligands located in the basement membrane (heparan sulphate proteoglycans: agrin and collagen 18) and developing muscle (Nucleolin). Disruption of ligand-PTPσ interactions affects axon guidance, although neither the role of PTPσ in neurons nor the effect of ligand binding on PTPσ activity is well understood. Further characterisation of PTPσ function remains difficult in the absence of an understanding of PTPσ biochemistry that would allow a functional assay to measure the effects of an experimental manipulation on PTPσ function.

PTPσ was shown to be dimeric using a combination of disulphide cross-linking and co-immunoprecipitation techniques. This dimeric form of PTPσ was principally cell surface localised according to its accessibility to trypsinisation. However, neither co-immunoprecipitation nor glutathione S-transferase (GST) pull-down techniques allowed the identification of proteins that interact with wild type or recombinant substrate-trapping PTPσ.

Secondly, H_2O_2 treatment of PTPσ-expressing cells induced the formation of reduction-sensitive, high molecular weight species. In contrast to the formation of PTPα dimers under the same conditions, this did not require the tyrosine phosphatase domain catalytic site cysteines. It may be possible to utilise the formation of high molecular weight species on non-reducing SDS-PAGE analysis as a proxy measure of PTPσ oligomerisation. Moreover, unlike co-immunoprecipitation it can be used on wild type and even endogenously expressed proteins.

Finally, the type IIa RPTP family consists of PTPσ, PTPδ and LAR. To allow the simultaneous disruption of type IIa RPTPs, chicken LAR was cloned from embryonic chick whole body mRNA and characterised.

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Abbreviations

AA/aa	Amino acids (Single letter code used throughout this thesis)
ADAM	"A disintegrin and metalloprotease"
AP	Alkaline phosphatase
β-Gal	β-galactosidase
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
C.elegans	*Caenorhabditis elegans*
CD	Cluster of differentiation
cDNA	Complementary DNA
CMV	Cytomegalovirus
COS7	African Green Monkey SV40-transformed kidney fibroblast cell line
CRYP	Chick receptor tyrosine phosphatase
D1	Membrane-proximal PTP domain in 2 domain RPTPs
D2	Membrane-distal PTP domain in 2 domain RPTPs
DEPC	Diethylpyrocarboanate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate(s)
DPTP	*Drosophila* PTP
Drosophila	*Drosophila melanogaster*
DSP	Dual specificity protein tyrosine phosphatase
$E_{81/90/S}$	Extracellular domain of type IIa RPTPs resulting from proteolytic cleavage
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
FBS	Foetal bovine serum (Heat inactivated)
FN3	Fibronectin type III domain
Fugu	*Takifugu rubripes*
GAP	GTPase activating proteins
GEF	Guanine nucleotide exchange factors
GPCR	G-protein coupled receptor
GST	Glutathione S-transferase
HA	Haemagglutinin

HEK 293T	Human embryonic kidney SV40 T antigen transformed cell line 293
HmLAr	*Hirudo medicinalis* LAR
HRP	Horseradish peroxidase
HSPG	Heparan sulphate proteoglycan
IAA	Iodoacetamide
Ig	Immunoglobulin domain
IG2	Anti-chicken PTPσ antibody directed against bacterial GST fusion protein containing the first two Ig domains (AA – 29-232)(Stoker et al., 1995a)
IPTG	Isopropyl-1-thio-β-D-galactoside
kDa	Kilodaltons
LAPCR	Long-accurate PCR
LAR	Leukocyte common antigen related protein
LB	Lysogeny broth
Liprin	LAR-interacting-protein.1 (LIP.1)-related-protein
MAM	Meprin/A5 /(RPTP)μ
mRNA	Messenger RNA
Mw	Molecular weight
$P_{75/85}$	Phosphatase domain of type IIa RPTPs resulting from proteolytic cleavage
PAGE	Polyacrylamide gel electrophoresis
PBS(T)	Phosphate buffered saline (with Tween 20)
PC	Protein convertase
PC6-3/PC-12	Rat phaeochromocytoma derived cell lines
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethyleneimine
Pfu	*Pyrococcus furiosus* DNA polymerase
PKC	Protein kinase C
PNGase F	Peptide N-glycosidase F
pSer	Phosphoserine
pThr	Phosphothreonine
PTP	Protein tyrosine phosphatase (domain/protein)
pTyr/pY	Phosphotyrosine
RAP	Receptor affinity probe
RGC	Retinal ganglion cell
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RPE	Retinal pigmented epithelium
rpm	Revolutions per minute
RPTP	Receptor protein tyrosine phosphatase
RT	Reverse transcription/transcriptase
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate (Lauryl sulphate)
SOB	Super-optimal broth
SOC	Super optimal (broth) catabolite repression
Taq	*Thermus aquaticus* DNA polymerase
TBS(T)	Tris buffered saline (with Tween 20)
TE	Tris-EDTA buffer
TGN	*Trans*-Golgi Network
TPA	Phorbol-12-myristate-13-acetate
VSV	Vesicular stomatitis virus
X-Gal	5-bromo-4-chloro-3-indoyl-galactoside

Acknowledgements:

For Samantha, without whom all of this could not have been done.

All of the work contained in this thesis was my own with the following exceptions. PC6-3 cells were transfected by Jennifer Nixon, the optimisation of GST-PTPσ fusion protein expression and purification was carried out in collaboration with Dr Clare Faux and some of the DNA sequencing was performed by Dr Caroline Paternotte.

In historical order, I would like to thank my parents for supporting my studies and Peter Freeland (Worth Abbey School), Drs Lesley Robson, Peter Shortland and Prof John Priestley (Queen Mary College, UL) for encouraging me to pursue my scientific studies. I must acknowledge the Worshipful company of Tallow Chandler's for funding my BSc degree and the UCL MB/PhD program for giving me the opportunity to undertake a PhD during my medical training. My supervisors, Dr Andy Stoker and Prof Andy Copp, provided invaluable support and guidance during my PhD studies, which were generously funded by the Child Health Research Appeal Trust. Profs Gordon Stewart and David Muller were always available for excellent advice.

Many other people helped me to complete this PhD: past and present members of the Molecular Medicine, Molecular Embryology, Medical Molecular, Developmental Biology and Neural Development Units at the Institute of Child Health 2000-2004. The following deserve particular mention, in no particular order: Peter Scambler, Carles Gaston-Massuet, Dave Faulkes, J-P Martinez-Barbera, Mohammed Hawadle, Caroline Paternotte, Clare Faux, Dan Alete, Hamid Hashemi, Antonio Matilla, Phillipa Mills, Sarah Ivins, Hannah Farmer, Emily Custard, Hugh Lemonde, Kevin Mills, "Harry" Papapetrou, Marilyn Monk, Hannah Lewis, Cathy Holding, Kate Sugars, Karen Matthews, John Estridge, Diane Gerrelli, Reena Salpekar, Simon Richardson, Radu Aricescu, Mike Hurley, Dianne Vaughan and anyone else I have forgotten.

Chapter 1: Introduction

All cellular organisms have the ability to detect and respond to extracellular stimuli and to fluctuations in their intracellular environment. This ability to receive stimuli and to generate appropriate responses to them may be termed homeostasis. Organisms can respond to environmental fluctuations by altering protein activity or location or by changing the profile of genes that they express. The major mechanisms used to regulate protein function are allosteric regulation and reversible protein phosphorylation. Of these, protein phosphorylation has perhaps evolved to be the most prominent and is a feature of all three domains of life: Archaea, Bacteria and Eucarya (Kennelly and Potts, 1999). The reasons for this are simple. Addition of a protein phosphorylation site in principle has only a low cost to the gene or organism concerned – the addition of a single or at most a few amino acid residues. However, the chemical properties of the phosphate group give a single phosphate group the power to disrupt protein structure significantly. This effect is most pronounced when the phosphorylation site is otherwise neutrally charged (e.g. serine, threonine and tyrosine at physiological pH). Moreover, phosphate groups readily form salt bridges with arginine residues, resulting in novel tertiary or quaternary protein structures (Johnson and Lewis, 2001).

1.1: Protein Tyrosine phosphorylation

The key role of protein phosphorylation in cellular processes means that the enzymes that mediate it must be tightly regulated (Levitzki and Gazit, 1995; Hunter, 2000; Alonso et al., 2004). A number of different protein phosphorylation systems exist in the three domains of life. These differ in the target amino acid and in catalytic mechanisms. The enzymes that catalyse protein phosphorylation and dephosphorylation are known as kinase and phosphatase enzymes, respectively. Although it was thought initially that some phosphorylation schema were confined to Eucarya and others found only in Bacteria or Archaea, it is now recognised that these enzyme families are relatively evenly distributed among all forms of life (Kennelly and Potts, 1996; Kennelly and Potts, 1999).

This thesis will be primarily concerned with tyrosine phosphorylation. Indeed, the work described in this thesis is part of an ongoing effort to understand the function of a single eucaryotic protein tyrosine phosphatase (PTP), PTPσ. Tyrosine phosphorylation together with serine/threonine phosphorylation represent the most important forms of protein phosphorylation seen in Eucarya. Although too large a subject to be dealt with

adequately here, a broad distinction may be drawn between these different kinds of protein phosphorylation (Barritt, 1992; Cohen, 2000; Krauss, 2001). In general, serine/threonine phosphorylation affects enzyme activity by causing changes in protein structure and the basal levels of serine/threonine phosphorylation are quite high. Phosphoserine and phosphothreonine binding domains that mediate protein-protein interactions are a relatively recent discovery (Yaffe and Elia, 2001; Yaffe and Smerdon, 2004). In contrast, tyrosine phosphorylation is much less common. Indeed, tyrosine phosphorylation accounts for less than 0.05% of the normal cellular phosphoproteome (pSer 92%, pThr 8%), although this may increase 6-fold in transformed cells (Hunter and Sefton, 1980). In contrast to serine/threonine phosphorylation, the main effect of tyrosine phosphorylation is not on enzyme activity although most tyrosine kinases require activation loop tyrosine phosphorylation for full activity (Hubbard and Till, 2000). Instead, tyrosine phosphorylation creates binding sites for adaptor or scaffold proteins that recognise phosphotyrosine using Shc- or Dok-like phosphotyrosine binding domains (PTBs) (Uhlik et al., 2005). In addition to mediating protein-protein interactions, the recognition of phosphotyrosine residues by PTBs in the same protein molecule can lead to profound alterations in tertiary structure that regulate enzyme activity (Xu et al., 1997).

1.1.1: Tyrosine phosphatase classification

The remainder of this introduction will focus on the class I or classical PTPs, in particular on the type IIa family of classical receptor PTPs (RPTPs), of which PTPσ is a member. However, the other families of tyrosine phosphatase enzymes will be briefly introduced and the reader is referred to recent reviews for more information on these proteins. Enzymes that can specifically dephosphorylate tyrosine residues may currently be divided into several phylogenetic and mechanistic categories (Alonso et al., 2004). The class I family of tyrosine phosphatases comprises classical cysteine-based tyrosine phosphatases and will be covered in more detail subsequently. The single vertebrate class II tyrosine phosphatase, low molecular weight PTP (LMPTP), is structurally related to bacterial arsenate reductases; its function is not clearly understood. The bacterial class II tyrosine phosphatases dephosphorylate autokinases involved in capsule synthesis. LMPTP can dephosphorylate a number of protein kinases. Homologues of this protein are highly conserved across all the domains of life (Alonso et al., 2004) and mutations in LMPTP are associated with many pathological conditions including atopy (Bottini et al., 2002). There are three vertebrate class III tyrosine phosphatases, CDC25A/B/C, which evolved from a bacterial rhodanese-like enzyme. These cell cycle regulating proteins dephosphorylate the

adjacent inhibitory tyrosine and threonine sites on cyclin dependent kinase (CDK)/cyclin kinase complexes resulting in cell cycle progression (Boutros et al., 2006). A fourth family of proteins has recently been shown to have tyrosine phosphatase activity. Class IV tyrosine phosphatases utilise a catalytic aspartate residue and exhibit a different catalytic mechanism from the cysteine-based class I-III proteins. The founder members of this sub-family are the 4 *Eyes absent* genes (Tootle et al., 2003; Rayapureddi et al., 2003; Li et al., 2003), which have been identified in a number of human genetic syndromes (Vincent et al., 1997; Abdelhak et al., 1997; Azuma et al., 2000; Rayapureddi et al., 2003). In contrast, class I to III tyrosine phosphatases all use a cysteine-based catalytic strategy and share a common active site structure (CX_5R) despite using different protein folds to generate this structure. Finally, histidine based acid phosphatases have also been shown to have physiological phosphotyrosine phosphatase activity (Meng and Lin, 1998; Fleisig et al., 2004). The catalytic mechanism of classical PTPs will be discussed in detail below.

1.1.2: Classical cysteine-based PTPs

The class I family of tyrosine phosphatases includes the classical tyrosine phosphatases and the VH1-like dual specificity phosphatases (DSPs) (Alonso et al., 2003). VH1-like DSPs form a diverse group of enzymes and may be divided into a number of sub-families. Confusingly, not all VH1-like DSPs actually have dual specificity. Mitogen-activated-protein-kinase (MAPK) phosphatases dephosphorylate threonine and tyrosine residues in the pTXpY motif of the activation loop of MAPKs (Farooq and Zhou, 2004). Slingshot phosphatases dephosphorylate an inhibitory phosphoserine on cofilin (Ohta et al., 2003; Huang et al., 2006). Phosphatase-found-in-regenerating-liver (PRL) PTPs are less well understood but may affect Rho family GTPases through a tyrosine phosphatase activity (Fiordalisi et al., 2006). CDC14 phosphatases have serine or threonine phosphatase activity and stabilise the CKI-1 cip/kip cyclin E inhibitor and cause cell cycle arrest (Saito et al., 2004; Koreth and van den Heuvel S., 2005). The myotubularin-related and PTEN phosphatases dephosphorylate inositol phospholipids and are implicated in a number of human genetic diseases (Wishart and Dixon, 2002; Laporte et al., 2003; Pendaries et al., 2003; Begley and Dixon, 2005). The remaining VH1-like DSPs are grouped together to form a heterogenous sub-family of "atypical" DSPs, some of which have mRNA 5'triphosphatase activity (Martins and Shuman, 2002; Changela et al., 2005). The atypical VH1-like DSPs generally lack the protein domains characteristic of other tyrosine phosphatases. It has been postulated that the atypical DSPs may be regulated using a

similar system to the combinatorial subunit principle utilised by serine/threonine phosphatases (Alonso et al., 2004).

1.1.3: Catalytic mechanism of classical PTPs

Cysteine-based tyrosine phosphatases catalyse the hydrolysis of the tyrosine phosphate monoester via a dissociative mechanism. Their catalytic mechanism has been the subject of extensive recent reviews and the reader is directed to these for further detail (Denu and Dixon, 1998; Barford et al., 1998; Kolmodin and Aqvist, 2001; Zhang, 2002; Zhang, 2003). However, there follows a brief overview of the PTP catalytic mechanism to allow the reader to understand some of the experimental approaches used in this thesis, particularly the use of "substrate-trapping mutations" (7.2.2, 7.2.3) and tyrosine phosphatase inhibitors. Throughout this thesis, sites involved in tyrosine phosphatase catalysis will be referred to by their position in the archetypal PTP1B tyrosine phosphatase domain. For instance, the PTP1B catalytic P-loop cysteine is located at amino acid 215. However, at their first introduction, mutations in PTPσ that affect residues involved in tyrosine phosphatase catalysis will also be referred to by their location within the chick PTPσ1 protein (cDNA: Genbank accession number L32780)(Stoker, 1994). All other PTPσ mutations will be referred to by their location within chicken PTPσ1 only.

Cysteine based tyrosine phosphatases are marked by the active site motif, CX_5R, which constitutes the P-loop. In all but CDC25 PTPs, this conserved sequence may be extended to $CX_5R(S/T)$. The P-loop forms the base of a fold in the phosphatase domain surface and is orientated such that the guanidium group of the P-loop arginine (R221) and the amide side groups of the P-loop backbone project into the cavity of this fold. This creates a positively charged pocket to receive the negatively charged phosphate group. Phosphate binding in the active site pocket is further stabilised by an adjacent α-helix dipole moment and the active site arginine. The depth of active site pocket is variable, being deeper in tyrosine-specific enzymes and shallower in those that can also accommodate the smaller serine or threonine groups (Jia et al., 1995). Although, tyrosine phosphatases were initially considered relatively non-specific with regard to their choice of substrate, in recent years this concept has been overturned. Numerous examples of PTP specificity have now been discovered and the complex structural and regulatory basis for this specificity is under investigation (Tonks and Neel, 2001).

The catalytic mechanism of these enzymes is quite different from that of classical serine/threonine phosphatases; they form a covalent phosphoenzyme intermediate (Guan and Dixon, 1991; Cho et al., 1992) and they do not require metal ions as cofactors. The reaction is nucleophilic and can be divided into two stages: formation and breakdown of the phosphoenzyme intermediate. The catalytic cysteine has a low pKa (4.6) (Zhang and Dixon, 1993) and hence is present in the thiolate form at physiological pH. This is achieved by interactions with the P-loop histidine (H214), with a second adjacent α-helix dipole moment, with main chain amide residues and via the formation of a hydrogen bond with the P-loop hydroxyl group (S/T222 – where present) (Zhang and Dixon, 1993; Denu and Dixon, 1998). In the first step, the active site cysteine acts as a nucleophile, attacking the phosphorus centre of the tyrosine phosphate group to form the phosphoryl-cysteine intermediate (PTP-Cys-PO_3) leading to release of the dephosphorylated substrate. Any mutation in the catalytic cysteine residue removes all catalytic activity although the resulting protein may still be able to bind substrates (Streuli et al., 1989; Streuli et al., 1990; Guan and Dixon, 1991).

The p-loop is surrounded by several other loops that contribute to catalysis and substrate recognition. The most important of these in catalysis is the WPD loop. This forms a mobile loop between two hinge regions that contains at its centre a highly conserved tryptophan, proline, aspartate sequence (W179, P180, D181). Following substrate binding the WPD tip of this loop moves over the substrate positioning D181 adjacent to the substrate (Jia et al., 1995). In the first stage of phosphatase catalysis D181 acts as a general acid donating a proton to the leaving group phenolic oxygen. This assists expulsion of the leaving group (Zhang et al., 1994b; Hengge et al., 1995). In the second stage of phosphatase catalysis D181 acts as a general base and extracts a hydrogen from a chelated water molecule, which is thus activated to act as the nucleophile in the second stage reaction (Wu and Zhang, 1996; Denu et al., 1996; Lohse et al., 1997). The water molecule is positioned by interactions with Q262 (Sarmiento et al., 1998; Zhao et al., 1998). The phosphoryl-cysteine intermediate is then hydrolysed by the activated water resulting in the restoration of the enzyme with the production of free inorganic phosphate.

As remarked above, mutations in the conserved P-loop cysteine destroy phosphatase activity but not substrate binding. Therefore, PTPs containing mutations at this site can be used to affinity purify PTP substrates. Owing to the two step catalytic mechanism of PTPs, a number of other mutations have been identified that remove catalytic activity or interfere with hydrolysis of the phosphoenzyme intermediate without

having significant effects on substrate binding. Collectively, proteins containing such mutations are known as "substrate-trapping" mutants and they have been used to identify a large number of potential PTP substrates (Blanchetot et al., 2005). This has been achieved primarily using co-immunoprecipitation and pull-down techniques. However, substrate-trapping mutants have also been used successfully in far-Western analysis and to identify PTP inhibitors.

The majority of studies have used one of two substrate trapping mutations, the C215S mutation in the P-loop (Sun et al., 1993; Furukawa et al., 1994; Jia et al., 1995) or the D181A mutation in the WPD loop (Flint et al., 1997). In addition, a number of other mutations have been useful in substrate trapping experiments although these have been less widely used (Q262A (Pannifer et al., 1998), S/T222A(Zhang et al., 1995; Agazie and Hayman, 2003; Merritt et al., 2006), C215D (Romsicki et al., 2003) and Y676F (PTPH1 nomenclature – in combination with D181A) (Zhang et al., 1999)). Finally, several studies have used combinations of these two mutations. In particular, the C215S/D181A (Agazie and Hayman, 2003) and the D181A/Q262A (Xie et al., 2002; Kontaridis et al., 2004) combinations have been widely used. Each trapping mutant has its own advantages and disadvantages. Indeed some mutations that work effectively in certain PTPs are not effective in others (Agazie and Hayman, 2003). However, the D181A mutation (alone or in combination with the Q262A mutation) is generally considered to be the most effective of the substrate trapping mutations (Garton et al., 1996; Aoki and Matsuda, 2000; Xie et al., 2002; Kolli et al., 2004). In addition to a greatly decreased catalytic rate, this mutant has a decreased K_m when compared to wild type protein. This is thought to be due to the loss of the potential electrostatic repulsion between the WPD loop aspartate and the incoming negatively charged phosphate group. In addition, there are general concerns that are a feature across the group. The most significant of these are the effects that such mutations have on overall protein structure, particularly with respect to substrate specificity and the interactions of these proteins with tyrosine phosphatase inhibitors, which will be considered subsequently. Although the substrate trapping mutants as a whole retain a low K_m, there is the possibility that these mutations may affect substrate binding. Indeed, although the C215S mutation retains the ability to recognise phosphotyrosine (Guan and Dixon, 1991), structural and kinetic analysis revealed differences between wild type PTP1B and the C215S mutant (Scapin et al., 2001). This resulted in the generation of the alternative C215D mutant which more closely resembled wild type protein but had a 7000-fold lower K_{cat} at physiological pH and may be of use in future substrate trapping

experiments (Romsicki et al., 2003). A final cautionary point can be made about the use of substrate trapping mutants with RPTPs. Although successfully used in RPTPs, the majority of substrate trapping experiments used intracellular PTPs. This is especially true of work carried out *in vivo* (Blanchetot et al., 2005).

This thesis uses several non-specific protein tyrosine phosphatase inhibitors to increase cellular phosphotyrosine levels. A general understanding of the mechanism of action of these agents is useful in discussing both the desired and unwanted effects that they have on RPTPs. Non-specific tyrosine phosphatase inhibitors may be divided into cysteine oxidising agents (e.g. peroxide) (Denu and Tanner, 1998) and those that compete with tyrosine phosphate for access to the active site (e.g. vanadate) (Trudel et al., 1991; Huyer et al., 1997). Pervanadate mediates its effects primarily through active site oxidation although it may retain some ability to compete with substrate for active site binding. In general, competitive inhibitors have a less marked effect on cellular phosphotyrosine levels and have the potential to interfere directly with the trapping of phosphatase substrates. On the other hand, oxidising inhibitors alter the conformation of the active site and so have the potential to perturb substrate binding. Original studies that showed that oxidising inhibitors could have reversible (e.g. cysteine to sulphenic acid (SOH) or irreversible effects (e.g. cysteine (SH) to sulphonic acid (SO_3H)) (Huyer et al., 1997; Denu and Tanner, 1998). The reversibility of oxidation has been suggested to be enzymatic as well as spontaneous. Recently, however it has been shown that PTPs are able to form a reversible cyclic structure (sulphenyl-amide) when exposed to oxidising agents that protects them from further oxidation and is also likely to block substrate binding (Salmeen et al., 2003). It therefore follows that substrate trapping mutants of PTPs, which lack the active site cysteine (e.g. C215S/D), should be relatively immune to the structural effects of oxidising PTP inhibitors. However in two domain RPTPs (Most RPTPs, for examples see 1.1.5), it is unclear whether the inhibitory effect of cysteine oxidising agents is mediated by or reflects changes in the membrane-proximal (D1), or membrane-distal (D2) tyrosine phosphatase domain or indeed both (Blanchetot et al., 2002a; Persson et al., 2004). Despite these concerns, the cellular level of tyrosine phosphorylation is so low that it is essential to artificially boost it in substrate trapping experiments (Blanchetot et al., 2005). In general, this may be achieved either with tyrosine phosphatase inhibition or with tyrosine kinase activation. Recently, novel inhibitors have been developed that are specific for individual PTPs (Puius et al., 1997; Reviewed in Zhang, 2003). These have considerable promise as both experimental tools and potentially in the future, as therapeutic agents.

1.1.4: The role of classical PTPs in human disease

The discovery of protein tyrosine kinases in 1980 was followed by a rapid recognition of the importance of tyrosine phosphorylation and tyrosine kinases in many physiological and pathological systems (Hunter and Sefton, 1980; Strawn and Shawver, 1998; Manning et al., 2002; Ostman et al., 2006; Vivekanand and Rebay, 2006). Initially there was an expectation that tyrosine phosphatases would very rapidly be identified as important players in human genetic diseases, particularly in oncogenesis where they might act as tumour supressor genes opposing tyrosine kinase oncogenes. However, nearly two decades after the identification of the first tyrosine phosphatase (Tonks et al., 1988; Charbonneau et al., 1989) this early promise has not yet been fulfilled. It is worth considering that the first PTP (Tonks et al., 1988; Charbonneau et al., 1989) was identified almost a decade after the first tyrosine kinase (Hunter and Sefton, 1980) and kinase-specific therapeutics have only recently reached the clinic (Giles et al., 2005; Tokunaga et al., 2006; Ventura and Nebreda, 2006; Larkin and Eisen, 2006). Therefore, the importance of PTPs in human disease has yet to be fully appreciated and manipulation of phosphatase activity may in the future yield promising therapeutic options. An understanding of the biochemistry of these proteins will no doubt help in this undertaking.

Mutations in a small number of PTPs have been shown to cause or be associated with human diseases including congenital and acquired conditions. The first identified mutation in a class I PTP shown to cause a congenital human disease was in a member of the cytoplasmic myotubularin inositol phospholipid phosphatase family (MTM1) (Laporte et al., 1996). Mutations in related genes cause phenotypically similar autosomal recessive Charcot-Marie-Tooth disease type 4B1 (MTMR2) and 4B2 (MTMR13/Sbf2) (Bolino et al., 2000; Azzedine et al., 2003; Senderek et al., 2003). It is thought that the similar phenotype of mutations in the active MTMR2 and inactive MTMR13 phosphatases may be conferred by virtue of deregulation of the activity of MTMR2. Interestingly, the wild-type MTMR13 is an inactive pseudophosphatase, which both interacts physically with dimeric MTMR2, increasing MTMR2 catalytic activity and competitively inhibits MTMR2 binding to lipid vesicles. This is suggestive of potential roles for the inactive membrane-distal D2 domains in type II RPTPs. In addition, Laforin, the protein product of the EPM2A gene is mutated in some cases of progressive myoclonic epilepsy of Lafora (Minassian et al., 1998). Finally, mutations in CD45 are one cause of X-linked severe combined immunodeficiency (SCID) (Cale et al., 1997; Kung et al., 2000). Interestingly, mice deficient for CD45 were known to have a SCID phenotype over 5 years before the first human CD45 mutations were

identified (Kishihara et al., 1993; Kung et al., 2000). Several human genetic syndromes are associated with PTP mutations. These include Noonan syndrome and LEOPARD syndrome (Shp2/PTPN11) (Tartaglia et al., 2001; Digilio et al., 2002). Mutations in the recently identified type IV PTP Eya (Eyes absent) family are responsible for several developmental syndromes (Abdelhak et al., 1997; Wayne et al., 2001; Schonberger et al., 2005).

In addition, disruption of PTP function has been implicated in many acquired conditions including autoimmune diseases, type II Diabetes Mellitus and cancer (Asante-Appiah and Kennedy, 2003; Stoker, 2005). PTP-associated autoimmune diseases include type I Diabetes Mellitus (IA2/PTPRN, IA2R/PTPRN-2, PTPN22/PTPN8) (Rabin et al., 1994; Morahan et al., 1998; Bottini et al., 2004), Systemic Lupus Erythematosis (PTPN22) (Kyogoku et al., 2004), Rheumatoid Arthritis (PTPN22) (Begovich et al., 2004; Carlton et al., 2005), Graves thyroiditis (PTPN22) (Smyth et al., 2004) and Multiple Sclerosis (CD45) (Jacobsen et al., 2000). PTPN22 in particular appears to play a prominent role in several autoimmune diseases (Criswell et al., 2005). Although it was expected that PTPs would be implicated in many cancers, no cancer syndrome has yet been associated with a PTP, although patients with PTPN22-related Noonan syndrome have an elevated incidence of certain cancers (Tartaglia et al., 2003). Although abnormalities in PTP expression are often detected in malignancies, it is difficult to know whether these mutations are significant or reflect genomic instability in neoplastic cells. The role of PTPs as both tumour supressor genes and oncogenes has recently been reviewed and is a rapidly developing area (Ostman et al., 2006). For example, DEP1, LAR, PTPγ, PTPτ, PTPN3, PTPN13 or PTPN14 are mutated in a quarter of colorectal cancers (Ruivenkamp et al., 2002; Wang et al., 2004). PTPτ mutations were the most frequently found and were associated with decreased phosphatase activity, suggesting a role for these PTPs as tumour suppressor genes (Wang et al., 2004). In contrast, activating PTPN11 mutations are oncogenic and the *H.pylori* CagA antigen, which is associated with gastric carcinoma, activates Shp2 (Tartaglia et al., 2001; Higashi et al., 2004; Bentires-Alj et al., 2004). A range of other pathogens and toxins have co-opted PTPs to mediate their effects and PTP polymorphisms may affect the effects of these agents (Krasnoperov et al., 2002; Fujikawa et al., 2003; Peek, Jr., 2003).

1.1.5: Classification of classical PTPs

The classification of PTPs has been an evolving subject. However, now that all of the classical PTPs in the human genome are thought to have been identified, this

classification is unlikely to change significantly. The most recent phylogenetic and structural PTP classification will be followed in this thesis (Andersen et al., 2004). This developed from an original, purely structural RPTP classification with little need for modification (Fischer et al., 1991; Brady-Kalnay and Tonks, 1995).

Classical tyrosine phosphatases are conventionally divided into receptor and non-receptor subtypes although several receptor sub-types contain members or isoforms that lack a transmembrane domain. There are nine non-receptor sub-types and eight receptor subtypes. As PTPσ is a receptor protein tyrosine phosphatase (RPTP), non-receptor PTPs will not be discussed in any further detail. For a classification of these enzymes, the reader is referred to the literature (Andersen et al., 2001b; Andersen et al., 2004).

The receptor protein tyrosine phosphatase subtype is divided into eight sub-groups based on their domain architecture and phylogenetic relationship (Brady-Kalnay and Tonks, 1995; Andersen et al., 2001b). There follows, a brief description of the domain architecture of the eight RPTP families together with representative vertebrate examples of each family. In addition, several invertebrate RPTPs are mentioned, as these will be referred to subsequently. The type I sub-group contain CD45 proteins, whose extracellular domain contains spectrin-like repeats in addition to fibronectin type III domains. PTPσ is a type II RPTP and this sub-group will be described in more detail below. The extracellular domain of type 3 RPTPs (e.g. PTPβ) contains only fibronectin type III domains. The *Drosophila* (*melanogaster*) genes DPTP4E and DPTP10D and the *Caenorhabditis elegans* *(C.elegans)* gene F44G4.8 are also members of the type III family. The Drosophila genes DPTP99A and DPTP52F and the *C.elegans* gene K04D7.04 are usually classified in the type III family. However, they exhibit significant differences from other type III RPTPs both in domain structure and in genomic organisation (Schindelholz et al., 2001). Type IV RPTPs (e.g. PTPα/ε) possess a very short, highly glycosylated extracellular domain. Both the type III and IV RPTP families include several non-receptor RPTP members. The extracellular domain of type V RPTPs (e.g. PTPγ/ζ) includes a large carbonic anhydrase-like domain. The type VI sub-group contains only the chicken CD45 orthologue. Type VII RPTPs (e.g. PCPTP1, STEP) contains both transmembrane and intracellular proteins. Finally, type IIX RPTPs (e.g. IA2/IA2β) are believed to be catalytically inactive proteins whose extracellular domain contains a RDGS adhesion recognition motif. Type I-II and type IV-VI RPTPs contain two intracellular tyrosine phosphatase domains, of which only D1 usually exhibits significant phosphatase activity due to the presence of a number of critical point mutations in D2 domains (Lim et al., 1998; Nam et al., 1999; Buist et al., 1999; Lim et al., 1999;

Krueger et al., 2003). However, it is becoming apparent that despite this, D2 domains play an important role in RPTP function. This includes effects on protein stability, D1 substrate specificity and overall RPTP regulation. In addition, D2 domains participate in key protein-protein interactions (Streuli et al., 1990; Wang and Pallen, 1991; Johnson et al., 1992; Debant et al., 1996; Wu et al., 1997; Kashio et al., 1998; Felberg and Johnson, 1998; Buist et al., 2000; Felberg and Johnson, 2000; Tsujikawa et al., 2001; Krueger et al., 2003; Persson et al., 2004; Wang and Johnson, 2005). In some systems, the residual activity of D2 domains may have a functional role (Garrity et al., 1999). All RPTPs with tandem phosphatase domains cluster together on phylogenetic analysis and all D2 domains form a separate off-shoot supporting the hypothesis that 2 phosphatase domain RPTPs resulted from the early intragenic duplication of a phosphatase domain followed by gene duplication (Hooft van Huijsduijnen, 1998; Andersen et al., 2001b). Type III (In general), VII and IIX RPTPs contain only a single intracellular tyrosine phosphatase domain; in type IIX RPTPs this is thought to be catalytically inactive (Lu et al., 1994; Drake et al., 2003). Indeed, these and other inactive PTP domains may function as atypical phosphotyrosine binding domains or via inhibitory interactions with other PTPs (Wishart and Dixon, 1998; Gross et al., 2002).

The type II RPTP subfamily contains proteins that cluster into two groups based on the absence (Type IIA) or presence (Type IIB) of an amino-terminal MAM domain (Meprin, A2, PTPμ). Otherwise, the extracellular domains of type IIA and IIB RPTPs are characterised by a number of immunoglobulin and fibronectin type III domains. Unfortunately, it is not unusual for RPTPs from different species to follow different and often inconsistent nomenclatures. Therefore, the synonymous names for each of the type II RPTPs are appended in parentheses below, after the name that is in most common use and which will be used throughout this thesis. The type IIA RPTP family contains 3 members in vertebrate species: PTPδ, PTPσ (CRYPα, LAR-PTP2, PTP-PS, PTP-P1, mPTPNU3, PTPT9a, PTPT9b) and LAR (RPTPf). The type IIB RPTP family contains four members: PTPκ, PTPλ (PCP2, PTPomicron, PTPpi, PTPi, PTPRO, PTPpsi but not chick PTPλ (CD45 orthologue)), PTPμ and PTPϱ (PTP-SL, PCPTP, PTPBR7, PC12-PTP1). The *Drosophila* gene DLAR and the *C.elegans* gene C09D8.1 are considered type IIa RPTPs. However, the structure of the DLAR gene shows no similarities to those of vertebrate type IIa RPTPs (O'Grady et al., 1994; Krueger et al., 1996). Therefore, it is unlikely that DLAR and human LAR are functional orthologues. Rather, DLAR and LAR are more likely to represent the independent development of cell adhesion molecule-like

RPTPs in the fly and vertebrate genomes from a common ancestral pool of domain types. The *Drosophila* gene DPTP69D and the *C.elegans* gene CLR-1 are often considered to be type IIa RPTPs despite differing from other type IIa RPTPs in domain structure and genomic organisation (Schindelholz et al., 2001). Flybase refers to DLAR as LAR and DPTP69D etc as PTP69D. However, to avoid confusion between vertebrate and invertebrate PTPs, the D prefix will be retained throughout this thesis.

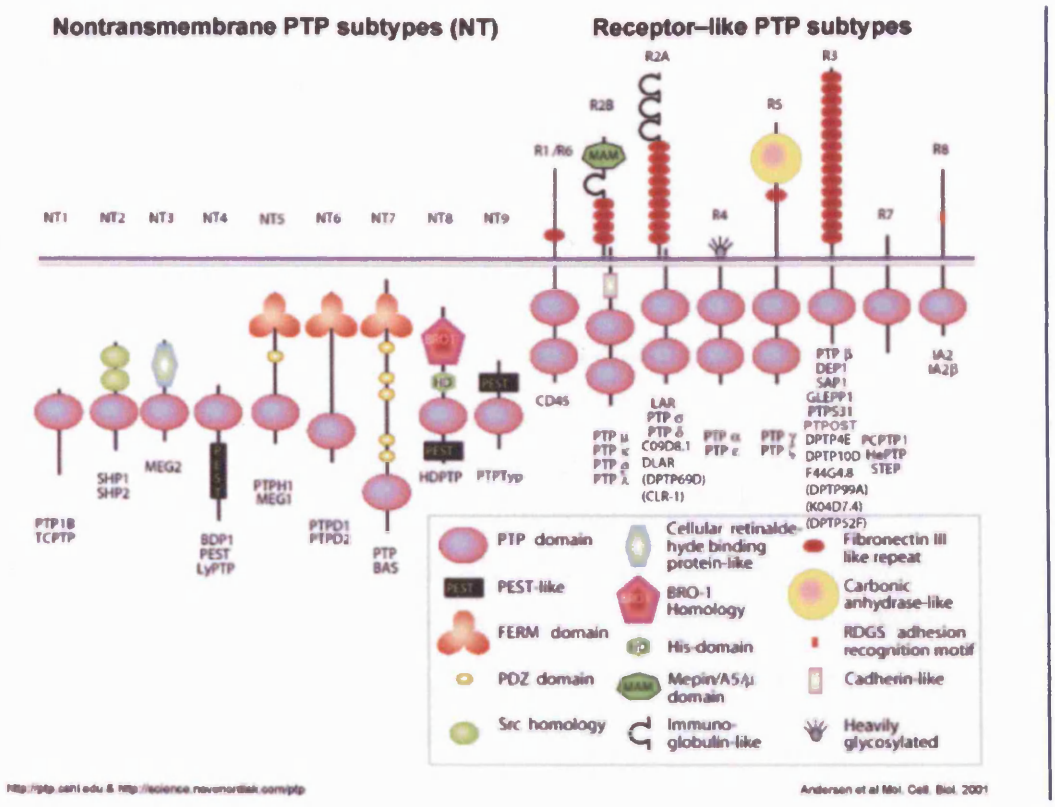


Figure 1.1: Classification of receptor protein tyrosine phosphatases

Modified from a published figure taken from the PTP web resource http://science.novonordisk.com/ptp/index.asp (Andersen et al., 2001b)

Schematic representation of PTP classification. Representative human examples of each family are included. In addition, several fly and *C.elegans* PTPs have been included as they will be included in subsequent discussions of PTP function. Those PTPs in brackets (e.g. CLR-1) are included in the most appropriate family although they may not have the canonical features of the family in which they are included. The 11 remaining *C.elegans* PTPs contain domains that evolved after separation of the lineage that gave rise to *C.elegans* from that which gave rise to vertebrates and insects

1.2: Type II RPTPs

The PTPσ gene is located on mouse chromosome 17 and human chromosome 19p13.3 (Yan et al., 1993; Wagner et al., 1994). Allelic forms have been reported in mouse (Yan et al., 1993), however there is little evidence for any association of PTPσ alleles with human disease. In contrast, although PTPδ is also not known to be associated with human disease, mutations in LAR have been associated with human disease (Jirik et al., 1992; Harder et al., 1995; Wang et al., 2004; Miscio et al., 2004). All three type IIa RPTPs have been disrupted in mouse models (Schaapveld et al., 1997; Elchebly et al., 1999; Wallace et al., 1999; Uetani et al., 2000). The phenotypes of these mutants and their implications for PTP function will be discusssed later (1.2.2). The general structure of the major PTPσ isoforms includes an extracellular domain containing a number of immunoglobulin (Ig) and fibronectin type III (FN3) domains and an intracellular domain containing one or two tyrosine phosphatase domains. As such, the domain architecture of PTPσ resembles that of several proteins known to be involved in cell-cell recognition and communication, which have been described as type I and type II members of the Ig superfamily (Vogel et al., 2003).

1.2.1: mRNA Expression

The type IIa RPTPs are conventionally thought to produce 2 major isoforms, one short (~6kb) and one long (~8kb) (Streuli et al., 1988; Yan et al., 1993; Mizuno et al., 1993; Walton et al., 1993; Sahin and Hockfield, 1993; Pan et al., 1993; Kaneko et al., 1993). The short isoform (e.g. CRYPα1; human PTPσ isoforms 3, 4; LAR-PTP B) corresponds to a transmembrane protein containing three amino-terminal Ig domains, followed by four FN3 domains and two intracellular tyrosine phosphatase domains. The longer isoform (e.g. CRYPα2; human PTPσ isoforms 1, 2; LAR-PTP A) includes an additional 4 FN3 domains between the 3rd and 4th FN3 domains of the shorter isoform (Mizuno et al., 1993; Stoker, 1994; Wagner et al., 1994; O'Grady et al., 1994; Mizuno et al., 1994). Antibodies directed against PTPσ have confirmed the presence of two major PTPσ protein species in tissues expressing both isoforms (Stoker et al., 1995a; Stoker et al., 1995b; Stepanek et al., 2005).

The expression pattern of PTPσ has been mapped by Northern and *in situ* analysis of mRNA expression and by immunological detection of PTPσ protein. In addition, it has recently become possible to analyse the expression pattern of PTPσ using a transgenic mouse line in which a β-galactosidase (β-Gal) gene has been inserted into the PTPσ locus

(Wallace et al., 1999; Meathrel et al., 2002). β-Gal detection in these animals has broadly confirmed studies that detect PTPσ mRNA or protein directly. PTPσ is quite widely expressed, but is one of few RPTPs to be preferentially expressed in neural tissue (Sahin et al., 1995). However, in addition to the two major PTPσ isoforms described above, a wide range of other species has been reported (Pan et al., 1993; Stoker, 1994; Rotin et al., 1994; Ogata et al., 1994; Zhang et al., 1994a). These are likely to represent alternatively spliced isoforms of PTPσ. Indeed, many RPTPs are known to produce multiple alternatively spliced isoforms including CD45. Alternative splicing of CD45 has significant implications for CD45 function (Xu and Weiss, 2002; Hermiston et al., 2003). Unfortunately, it is unlikely that the probes used in previous experiments will have detected all PTPσ isoforms and only a limited number of studies have explicitly discriminated between even the two major species. This is potentially of great significance for instance, mRNA for the recently described LAR FN5C isoform is as abundant as that for the full length isoform but was not detected in previous studies due to probe design (Yang et al., 2003). These outstanding issues must be borne in mind when considering the currently available studies of PTPσ isoforms and expression pattern.

1.2.1.1: Isoforms generated by mRNA alternative splicing

The alternative splicing of PTPσ has not been studied in any detail. However, a large number of alternatively spliced isoforms have been reported for the remaining type IIa RPTPs. There is evidence that the expression of these isoforms is developmentally and spatially regulated (Pan et al., 1993; Longo et al., 1993; Stoker, 1994; Rotin et al., 1994; Ogata et al., 1994; Mizuno et al., 1994; Wang et al., 1995; Sahin et al., 1995; Zhang and Longo, 1995; Pulido et al., 1995b; Honkaniemi et al., 1998; Zhang et al., 1998; Ledig et al., 1999b; Chilton and Stoker, 2000; Zhang et al., 2003; Yang et al., 2003). Where this has been examined, homologous isoforms are found for all three genes (Stoker, 1994; O'Grady et al., 1994; Zhang and Longo, 1995; Pulido et al., 1995a). In addition, comparison of the structure and sequence of these genes suggests that findings in one gene are likely to be conserved across the family (O'Grady et al., 1994). These genes produce isoforms from two major kinds of alternative splicing events.

Firstly, the gene structure of the type IIa RPTPs means that domain and exon boundaries largely coincide (O'Grady et al., 1994). Therefore, individual domains can be precisely excised to produce alternative isoforms with different numbers of Ig, FN3 or PTP domains (Mizuno et al., 1993; Pan et al., 1993; Stoker, 1994; Zhang and Longo, 1995;

Pulido et al., 1995a). Examples include the short and long major isoforms described above. In addition, soluble isoforms lacking the PTP domains and isoforms consisting only of Ig or FN3 domains have also been reported (Stoker, 1994; Yang et al., 2003). Although ligand-binding differences have been reported for the two major PTPσ isoforms (Haj et al., 1999; Chilton, 2000; Sajnani-Perez et al., 2003), the functional significance of many of these isoforms is unknown. In addition, the two isoforms of the *C.elegans* type IIa RPTP, PTP-3, have distinct roles in axon guidance and synaptogenesis (Ackley et al., 2005). Computer predictions (2.6.3) suggest that the major alternatively spliced isoforms of CRYPα do not differ in potential tyrosine sulphation sites (Kehoe and Bertozzi, 2000). However, CRYPα2 has twice the predicted N-glycosylation (4 vs 2) and O-glycosylation (2 vs 1) sites found in CRYPα1 (Yan et al., 1993; Stoker, 1994). Experimental studies will be required to determine whether these predicted differences are real. However, given the importance of differential glycosylation in CD45 function, this difference in the glycosylation potential of PTPσ isoforms is of interest (Xu and Weiss, 2002).

Secondly, a number of small alternatively spliced exons have been reported for PTPδ and LAR, which add short peptide sequences rather than whole domains. Pulido et al reported 3 small PTPδ mini-exons (me): meA introduces 9 amino acids within the 2nd Ig domain, meB introduces 4 amino acids between the 2nd and 3rd Ig domains; finally, meC introduces 9 amino acids into the 5th FN3 domain and is identical to LASE-c (See below) (O'Grady et al., 1994; Pulido et al., 1995a). Several alternatively spliced forms of LAR have been reported (LAR alternatively spliced element – LASE) (O'Grady et al., 1994; Zhang and Longo, 1995). The LASE-a exon introduces 11 amino acids just upstream of the juxtamembrane wedge. The LASE-a coding sequence includes a juxtamembrane cysteine residue which may contribute to disulphide bond formation between native RPTP monomers (Chapter 4; van der Wijk et al., 2004). This exon also contains two conserved serine residues that play a key regulatory role in PTPα (Tracy et al., 1995; Stetak et al., 2001). LASE-b introduces four amino acids just after the transmembrane domain. LASE-c is equivalent to PTPδ meC and equivalent isoforms are found in LAR, PTPσ and PTPδ (O'Grady et al., 1994; Zhang and Longo, 1995). The LASE-c/meC insert has been shown to mask a laminin-nidogen binding site in the fifth FN3 domain and it is also included in a homophilically binding isoform that supports neurite outgrowth (O'Grady et al., 1998; Yang et al., 2003; Yang et al., 2005). In contrast to the other small alternatively spiced exons, LASE-d and LASE-e are associated with major architectural changes. The LASE-d exon introduces a stop codon between FN3 domain number 5 and 6, resulting in a

truncated soluble protein (Zhang and Longo, 1995). It is interesting to speculate why type IIa RPTPs might have developed the ability to generate secreted forms both from proteolysis of transmembrane proteins and alternative splicing (Streuli et al., 1992). LASE-e has been identified in association with the LASE-c and LASE-d exons. Isoforms including the LASE-e exon contain a novel start codon just upstream of the LASE-c insert in FN3 domain 5. The association with LASE-d results in a small (11 kilodalton (kDa)) secreted protein (LARFN5C) mainly comprised of the C-terminal portion of FN3 domain 5. This secreted motif contains a homophilic binding site that is able to mediate LARFN5C tetramerisation and also binding to full-length forms of LAR. Despite its effects on the intact domain, the presence of the LASE-c exon does not disrupt the neurite outgrowth promoting effect or the homophilic binding site of the c-terminal region of FN3 domain 5. (Yang et al., 2003; Yang et al., 2005). Due to their similar properties, it is likely that the laminin-nidogen binding site will prove to be closely related to the FN5C homophilic adhesion site. The CRYPα1 and -α2 cDNAs are LASE-a, d, e and meA, B negative and LASE-b positive. The Longer CRYPα2 cDNA is LASE-c/meC negative (Stoker, 1994; O'Grady et al., 1994; Zhang and Longo, 1995; Pulido et al., 1995a; Yang et al., 2003). Therefore, they lack the currently identified homophilic and ECM binding sites reported above.

The presence of currently undescribed PTPσ isoforms should be borne in mind when studying the reported PTPσ expression data. However, several points can be made with the available data on the major large (3 Ig, 8, FN3, 2 PTP) and small (3 Ig, 4 FN3, 2 PTP) isoforms. PTPσ is expressed from an early developmental stage (Hara et al., 2003). It is tempting to speculate that type II RPTPs might have a conserved role in early vertebrate and invertebrate development (Fitzpatrick et al., 1995; Bateman et al., 2001; Frydman and Spradling, 2001; Harrington et al., 2002; Chin-Sang et al., 2002a; Hinton et al., 2003). In addition, PTPσ expression can be detected in the visceral endoderm and yolk sac of early embryos (Sousa-Nunes et al., 2003).

1.2.1.2: Expression patterns

Northern analysis shows that whilst the long isoform is expressed in many tissues, expression of the short isoform is limited to neural tissue (Yan et al., 1993; Walton et al., 1993; Sahin and Hockfield, 1993; Stoker, 1994; Rotin et al., 1994; Zhang et al., 1994a; Wang et al., 1995; Sahin et al., 1995; Kim et al., 1996; Ranjan and Hudson, 1996; Schaapveld et al., 1998; Haj et al., 1999; Elchebly et al., 1999; Ledig et al., 1999b; Chilton

and Stoker, 2000; Johnson and Holt, 2000; Sajnani-Perez et al., 2003; Sapieha et al., 2005). PTPσ expression is low in the mature animal except in discrete regions of the central nervous system. However, many tissues express PTPσ at high levels during development for example, the central and peripheral nervous system, many neural crest derived tissues, endodermal derivatives such as the liver and mesodermal derivatives including muscle (smooth, cardiac and skeletal) (Yan et al., 1993; Walton et al., 1993; Sahin and Hockfield, 1993; Stoker, 1994; Rotin et al., 1994; Zhang et al., 1994a; Sahin et al., 1995; Norris et al., 1997; Ostenson et al., 2002; Lajus and Lang, 2006). Several studies report conflicting data regarding the expression of PTPσ in different tissues. This may be attributable to differences in the choice of probes with differing abilities to recognise PTPσ isoforms. Finally, the timing of PTPσ expression during development varies between species. This is likely to reflect differences in the developmental ages of experimental model species at parturition.

In non-neural tissue, which expresses only the larger PTPσ isoform, expression is upregulated in actively proliferating and differentiating epithelial cells but reduced in quiescent cells (Rotin et al., 1994; Kim et al., 1996). This is reminiscent of the effect of increasing cell-cell contact on PTPσ expression (Celler et al., 1995). In contrast, PTPσ expression peaks twice during neural development. During embryonic development, PTPσ expression in most neural tissue changes from a situation where the larger major isoform predominates to one where the shorter isoform is the more common (Yan et al., 1993; Sahin and Hockfield, 1993; Stoker, 1994; Wang et al., 1995). The larger isoform is expressed most strongly in the proliferating neuroepithelium whereas the shorter isoform is found predominantly in migrating and differentiating mantle zone cells. Finally, towards the end of embryonic development, PTPσ expression changes again from the relatively widespread developmental expression pattern to a picture more reminiscent of the adult with localised areas of high signal (Stoker, 1994; Wang et al., 1995; Sahin et al., 1995).

In the adult organism, PTPσ expression is significantly lower than in the developing embryo. Expression in non-neuronal tissues is minimal. In neural tissues, both neurons and myelinating oligodendrocytes and Schwann cells express PTPσ. However, both the long and in particular the short isoform are expressed strongly in discrete areas within the central nervous system although the overall level is lower than during embryonic development. The highest levels are seen in the hippocampus, rhinencephalon/paleopallium and cerebellum. In the hippocampus, PTPσ is expressed in the granule cells of the dentate gyrus and in the cells to which they project, the pyramidal

cells of the hippocampus proper. PTPσ is expressed in olfactory neuroepithelium, in mitral and granule cells in the olfactory bulb and in the olfactory cortex. Expression is also maintained in the pyramidal cells and internal granule layer of the cerebellum (Walton et al., 1993; Sahin and Hockfield, 1993; Pan et al., 1993; Rotin et al., 1994; Wagner et al., 1994; Ogata et al., 1994; Zhang et al., 1994a; Wang et al., 1995; Sahin et al., 1995; Kim et al., 1996; Norris et al., 1997; Haworth et al., 1998; Ostenson et al., 2002; Meathrel et al., 2002; McLean et al., 2002; Thompson et al., 2003; Batt et al., 2003). The areas that continue to express PTPσ in the adult brain share several interesting characteristics. The olfactory and gustatory neuroepithelium are the only neuronal tissues that are known to be continually regenerated throughout life (Beidler and Smallman, 1965; Graziadei and Graziadei, 1979). The lifespan of individual olfactory receptor neurons is on the order of 20-30 days (Samanen and Forbes, 1984). New cells are continually produced that elaborate an axon, which fasciculates with the olfactory nerve before passing through an olfactory ensheathing cell sheath, to form a synapse at the appropriate site in the olfactory bulb (Doucette et al., 1983). Similarly, the subventricular zone-olfactory bulb system and the granule cell layer of the dentate gyrus are the only regions of the mammalian central nervous system that continue to exhibit neurogenesis in the adult animal (Altman and Das, 1965; Altman, 1969; Pencea et al., 2001; Kempermann, 2002). In addition, the hippocampus is an area known to show high levels of synaptic plasticity, while neurogenesis in the adult hippocampus may play a role in memory processing (Martin et al., 2000; Lamprecht and LeDoux, 2004). Similarly, cerebellar plasticity may be involved in motor learning (Ito, 2001; De Zeeuw and Yeo, 2005).

The expression pattern of PTPσ has been most extensively described in the developing visual pathway and spinal cord of the chick embryo. Where examined, the pattern of PTPσ expression in the chick is matched in the mouse visual pathway (Sapieha et al., 2005). For a number of reasons, the visual pathway is among the most well described models of axon guidance (Mey and Thanos, 2000; Thanos and Mey, 2001). In particular, the generation and maintenance of the retinotopic map throughout the visual pathway has been extensively described both anatomically and molecularly (Mey and Thanos, 2000; Thanos and Mey, 2001).

Neuroepithelial development (primary neurogenesis) has 4 stages: proliferation of neuroblasts, followed by their vertical migration, the formation of processes and synapse formation and finally, programmed cell death and process pruning (Gilbert, 1997). The development of the chick retinotectal system will be described in more detail below.

Briefly, the inner layer of the invaginated optic cup forms the neural retina, proliferating to form a laminated structure comprising the seven major retinal cell types (Sadler, 1995; Gilbert, 1997; Masland, 2004). Although the birthdates of retinal cell types overlap, retinal ganglion cells (RGCs) are among the first cells to be produced and migrate furthest, being followed by the cells of the more external retinal layers (Young, 1985; Prada et al., 1991; Hu and Easter, 1999). RGCs then elaborate axons that run along the vitreal basement membrane towards the optic cup. In the chick, the first RGC axons reach the tectum at E6. The RGC dendritic arbor develops about a week after the axon reaches the tectum in parallel with the differentiation of the cells of the inner nuclear layer.

The neural retina differs from other neural tissues in the pattern of PTPσ isoforms it expresses. At all developmental stages, the long form of PTPσ predominates in the retina in contrast to the shorter isoform that is more common in other neural tissues (Stoker, 1994; Stoker et al., 1995b). Early in development, PTPσ is expressed in the proliferative zone and widely in migrating neuroblasts in the mantle zone or inner neuroblastic layer. However, as retinal lamination proceeds, it becomes apparent that PTPσ expression is highest in post-mitotic neurons of the RGC and inner nuclear layers (INL). However, isoform specific studies indicate that initially, RGCs only express the short PTPσ isoform. RGC expression of the longer isoform is upregulated after RGC axons reach the tectum. Although both isoforms are expressed in the developing INL, the short isoform is particularly strongly expressed in cells with the characteristics of amacrine cells. In the mature retina, a similar pattern of expression is seen, however the level of expression is lower (Stoker, 1994; Haj et al., 1999; Ledig et al., 1999b; Johnson and Holt, 2000; Sapieha et al., 2005). In early retinal development in contrast, PTPσ protein is predominantly found in the layer of RGC axons adjacent to the developing vitreous. There is also some weak staining of processes that span the inner plexiform layer. As development proceeds, PTPσ is also found in the area of the developing vitreal basement membrane and the staining of inner plexiform processes becomes more marked. In late development, PTPσ protein levels may be reduced but show the same pattern of localisation (Stoker et al., 1995b; Ledig et al., 1999b). Finally, RGC axons are not myelinated until late in development. The oligodendrocytes of the mature optic and other nerves express PTPσ (McLean et al., 2002; Sapieha et al., 2005). In summary, PTPσ protein is located on the axons of retinal cells as they extend processes to contact other neurons and changes in isoform expression are associated with the progress of neurite extension.

The optic tectum develops in parallel with the retina to form a complex structure with 15 histologically defined layers containing at least 20 cell types (Huber and Crosby, 1933; Mey and Thanos, 2000). Briefly, the proliferating tectal neuroepithelium undergoes 2 phases of neuroblast migration. The first wave of migrating cells produces the *stratum griseum centrale* (SGC) and the *stratum griseum periventriculare* (SGP). The large SGC cells are the major tectal efferents. These cells elaborate axons that form the circumferentially running tectobulbar tract of the *stratum album centrale* (SAC) that runs over the SGC. The second wave of neuroblast migration occurs in parallel with the arrival of retinal axons at the anterior tectal pole. These cells migrate above the SAC and subsequently form the multilaminar *stratum griseum et fibrosum superficiale* (SGFS) region in such a way that RGC axons are never in direct contact with the SAC axon bundles. Unusually, this second wave of migration generates the deeper SGFS layers before the superficial SGFS layers, in the reverse manner to that seen in most other developing neuroepithelia. RGC axons do not immediately enter the tectum, but rather course along its surface to a topologically appropriate location. The arrival of new axons occurs in such a way that they always run on top of their predecessors such that growth cones remain in contact with the radial glia endfeet in the pial basement membrane. Almost a week after they arrived at the anterior tectum, the first RGC axons enter the tectum and pass into the SGFS layers to form defined terminal arbors of a predictable size and shape. Axons exhibit multiple course corrections and their trajectories and fasciculation becomes more refined with time.

Expression of PTPσ in the chick optic tectum may be divided into two areas. The long isoform is expressed from the earliest stages in the ventricular zone whilst these cells are still proliferating. In contrast, the short isoform is expressed in differentiated neuroblasts as these appear and form the definitive cellular layers of the tectum (Stoker, 1994; Haj et al., 1999). As in the retina, PTPσ protein is predominantly found in fibre rather than cellular layers. In early development, before the RGC axons arrive, PTPσ signal is prominent only in the presumptive tectobulbar tract that will later form the SAC. Later in development, PTPσ is detectable in the more superficial plexiform SGFS layers, into which the RGC axons will migrate. As reported above, RGC axons express PTPσ. PTPσ maintains this pattern of expression albeit at a lower level in the mature tectum (Stoker et al., 1995b; Ledig et al., 1999b). In the tectum therefore, PTPσ is expressed in the tectofugal fibre tracts and also in some of the areas targeted by ingrowing RGC axons.

The development of spinal motor neurons in the ventral horn of the chick lumbar spinal cord has been extensively reviewed elsewhere. Briefly, a combination of dorsoventral

and regionalising signals specify the generation of motor neurons expressing members of the *LIM* (Lin11-Isl1-Mec3) homeodomain transcription family (Price and Briscoe, 2004). In the chick, motor neurons are born between Hamilton-Hamburger (HH) stage 15 and stage 23 having previously been specified with regard to their targets (Hollyday and Hamburger, 1977; Matise and Lance-Jones, 1996).

As in the tectum, in early spinal cord development, PTPσ is expressed in the proliferating cells of the ventricular zone. As development proceeds, PTPσ expression is extended to all the ventral horn motor neurons. In addition, a significant proportion of cells scattered through the spinal cord express the short isoform of PTPσ at levels that are demonstrably lower than are those seen in proliferating ventricular zone cells or motor neurons. Isoform-specific studies confirmed that as in the tectum, proliferating ventricular zone cells express the long isoform of PTPσ and the differentiating cells that elaborate long axons, in this case motor neurons, express the short form. In addition, PTPσ is expressed on axons within the fibre tracts of the ventral commissure and the dorsal funiliculus (Sahin and Hockfield, 1993; Stoker, 1994; Stoker et al., 1995a; Chilton and Stoker, 2000).

In summary, PTPs are expressed in a wide range of developing tissues, particularly in the nervous system. In the adult brain, PTPσ is restricted to areas that exhibit ongoing neurogenesis or plasticity. In addition, the expression of particular isoforms is developmentally regulated such that the large and short PTPσ isoforms are associated with proliferating and differentiating cells respectively. Finally, it is intriguing that PTPσ is often expressed both on growth cones and on their targets. For instance, PTPσ is expressed on RGC and motor neuron axons and in their targets, the optic tectum and muscle respectively (Stoker, 1994; Stoker et al., 1995b; Schaapveld et al., 1998; Ledig et al., 1999b; Chilton and Stoker, 2000; Johnson and Holt, 2000). This mimics the pattern of DLAR expression on *Drosophila* photoreceptor axons and motor axons and on their targets, the optic lobe lamina and medulla and peripheral muscle (Clandinin et al., 2001; Fox and Zinn, 2005) and may therefore represent a common feature of type IIa RPTP function.

1.2.2: Functional studies of type IIa RPTPs

The expression pattern of PTPσ in the developing and adult brain strongly suggests that continued expression of the short PTPσ isoform in the adult brain is limited to regions known to exhibit significant plasticity or in the case of the olfactory neuroepithelium, ongoing proliferation. In addition, the domain structure of PTPσ is reminiscent of many

cell adhesion and axon guidance molecules. Axon guidance is mediated by a combination of multiple partially redundant long- and short-range guidance cues, which act at a large number of axon guidance choice points. These cues affect the rate or direction of growth cone migration and the formation or separation of axon fascicles by individual growth cones (Tessier-Lavigne and Goodman, 1996; Desai et al., 1996). PTPσ is a transmembrane protein that undergoes ectodomain shedding and so is well suited to acting as a contact or diffusible axon guidance molecule. Studies have shown that tyrosine phosphorylation plays an important role in the developmental migration of axon growth cones to their appropriate targets and the targeted regeneration of severed axons (Desai et al., 1997b). Furthermore, immunological studies confirmed that PTPσ protein is indeed expressed in migrating axon growth cones like type IIa RPTPs in other species (Stoker et al., 1995a; Biswas et al., 2002). Studies in *Drosophila* have confirmed that RPTPs including the type IIa RPTP DLAR are involved in the guidance of photoreceptor (Garrity et al., 1999; Newsome et al., 2000a; Maurel-Zaffran et al., 2001; Clandinin et al., 2001), motor neuron (Desai et al., 1996; Krueger et al., 1996; Desai et al., 1997a; Sun et al., 2001; Schindelholz et al., 2001) and commissural axon growth cones (Sun et al., 2000a). These studies have described a range of axon guidance phenotypes including stall, parallel bypass and fusion bypass and have shown that individual RPTPs can act together or in opposition at specific choice points in the path of a growing axon (Desai et al., 1997a; Sun et al., 2001). Similarly, type IIa RPTPs are involved in axon guidance, neuroblast migration and the formation of an orchestrated pattern of parallel neurite processes through mutual repulsion and sibling avoidance in the roundworm, *C.elegans*, and the medicinal leech, *Hirudo medicinalis* (Gershon et al., 1998a; Gershon et al., 1998b; Baker and Macagno, 2000a; Baker et al., 2000b; Baker and Macagno, 2000b; Harrington et al., 2002; Chin-Sang et al., 2002b; Chang et al., 2004; Ackley et al., 2005). However, a number of questions remain concerning RPTP function in axon guidance. It is unclear why when many of these proteins are expressed on the majority of axons, only a subset of axons are affected in mutants. Similarly, it is unclear whether the RPTPs act primarily as ligands or receptors and whether the phosphatase activity of these proteins is essential for their function. Finally, it is not certain whether these proteins affect axon guidance through effects on the rate of neurite outgrowth, growth cone direction or fasciculation.

Mice in which the PTPσ locus has been targeted and disrupted by insertion of a recombinant cassette (PTPσ knockouts) exhibit significant perinatal mortality and abnormalities in physical and behavioural development (Elchebly et al., 1999; Wallace et al.,

1999). However, there was phenotypic variability between the two PTPσ knockout lines and many of the developmental abnormalities appeared to correct with age if pups were supported through the neonatal period (Elchebly et al., 1999; Wallace et al., 1999; Batt et al., 2002; Meathrel et al., 2002). It was elegantly demonstrated that neonatal survival could be drastically improved with neonatal growth hormone (GH) supplementation of PTPσ -/- pups (Batt et al., 2002). As expected given the correction of many aspects of the PTPσ -/- phenotype with age, GH supplementation also reverses several of the histopathological features of the PTPσ -/- phenotype including the abnormalities of the pituitary and endocrine pancreas (Batt et al., 2002). It is unclear whether other aspects of the PTPσ phenotype respond to GH supplementation in this way. In summary, it appears that the phenotype of the PTPσ -/- mice reflects developmental delays in several important structures including the pituitary and endocrine pancreas. However, although the two PTPσ knockout lines have similar phenotypes, there are significant differences between them. The remaining type IIA RPTPs have been disrupted in a similar way (Skarnes et al., 1995; Schaapveld et al., 1997; Uetani et al., 2000). Unfortunately, there is evidence that these lines can express residual forms of the disrupted gene (Yeo et al., 1997; Yang et al., 2003). It may be noted that the approach used to generate the Elchebly line would suggest that residual PTPσ activity would most likely be in the form of extracellular domain protein. In contrast, the targeting strategy adopted to create the Wallace line implies that residual PTPσ activity would be in the form of catalytic domain protein. These differences may account for discrepancies in the phenotypes of the two PTPσ knockout mice as may differences in the genetic background of the two lines.

All three type IIa RPTPs have been disrupted using gene targeting approaches (Skarnes et al., 1995; Schaapveld et al., 1997; Elchebly et al., 1999; Wallace et al., 1999; Uetani et al., 2000). Although each type IIa RPTP knockout has a distinct phenotype, there are suggestions from their phenotypes that in some tissues, these genes may play similar roles and in others that they may counteract each other. Like PTPσ -/- mice, LAR -/- mice display abnormal peripheral nerve regeneration (Johnson et al., 2001; McLean et al., 2002; Thompson et al., 2003; Lorber et al., 2004; Lorber et al., 2005; Sapieha et al., 2005; Kirkham et al., 2006). In addition, LAR -/- mice exhibit abnormalities in the development of lactation and also alterations in septohippocampal innervation that are associated with impaired spatial learning (Schaapveld et al., 1997; Yeo et al., 1997; Van Lieshout et al., 2001). Like PTPσ -/- mice, PTPδ -/- mice show growth retardation and increased neonatal mortality. The increased neonatal mortality seen in the PTPδ -/- pups is due to delays in

neuromuscular maturation. However, like LAR -/- mice PTPδ -/- also exhibit impaired hippocampal long term potentiation (LTP), which is associated with impaired spatial learning in these animals (Uetani et al., 2000). The expression pattern of PTPσ and PTPδ overlap in many areas, whereas the expression pattern of LAR is more often reciprocal to that of PTPδ/σ. In particular, PTPσ and PTPδ are both highly expressed in the developing nervous system whereas LAR is only expressed at low levels in this area (Yan et al., 1993; Mizuno et al., 1993; Longo et al., 1993; Rotin et al., 1994; Katsura et al., 1995; Sommer et al., 1997; Schaapveld et al., 1998). In addition, RNA interference studies suggested that PTPσ and PTPδ have overlapping functions in the guidance of specific motor axons and synaptogenesis (Dunah et al., 2005; Stepanek et al., 2005). Therefore, PTPσ and PTPδ -/- mice were intercrossed to determine whether PTPσ and PTPδ exhibit a degree of functional redundancy during nervous system development. The most striking findings in double mutant mice (PTPσ -/-; PTPδ -/-) are significant abnormalities in phrenic nerve development (Uetani et al., 2006). It is unclear at this stage whether this phenotype reflects a primary neuronal or muscular defect. Double mutant motor neurons extend to the periphery normally but then retract having failed to interact appropriately with their muscle target. Motor neurons are then lost, probably due to a lack of peripheral target-derived trophic support (Sendtner et al., 2000). A similar final phenotype is seen with DLAR, DPTP69D or N-cadherin mutant photoreceptors which terminate early in the R8 layer of the optic lobe lamina (Newsome et al., 2000a; Maurel-Zaffran et al., 2001; Clandinin et al., 2001; Lee et al., 2001). However, the double mutant phenotype is also associated with impaired myotube development, which may alternatively imply that the double mutant phenotype reflects a primary muscle defect. Indeed, PTPσ and PTPδ are both expressed in muscle (Schaapveld et al., 1998; Sajnani-Perez et al., 2003). In addition several mice, which are deficient in molecules that have been implicated in PTPσ function have significant muscle phenotypes (Debant et al., 1996; Gautam et al., 1996; Bateman et al., 2000; O'Brien et al., 2000).

In addition to its role in neural development, PTPσ -/- animals revealed a continuing role for PTPσ in mature neurons. Nerve injury and culture models show that the rate of neurite outgrowth is increased from PTPσ -/- neurons when compared to wild type neurons and that this is associated with more rapid functional recovery (McLean et al., 2002; Thompson et al., 2003; Sapieha et al., 2005; Kirkham et al., 2006). These studies suggest that PTPσ functions to reduce the rate of axon extension from wild type cells. Moreover, overexpression of a putative dominant negative PTPσ protein in cultured RGCs

increases neurite outgrowth (Johnson et al., 2001). However, although PTPσ-/- axons regenerate more quickly, they also exhibit more pathfinding errors (McLean et al., 2002). This may reflect a role for the regulation of growth cone velocity in the correct interpretation of axon guidance cues. However, it is unclear whether PTPσ acts as a general inhibitor to neurite outgrowth or whether it interprets inhibitory gradients of other molecules. Interestingly, early findings suggested that PTPσ mRNA is increased after nerve injury. This would be compatible with PTPσ playing an important role in the regeneration of accurate connections. More recent studies have failed to show any change in PTPσ protein levels (Haworth et al., 1998; Thompson et al., 2003; Sapieha et al., 2005). However these protein studies compared PTPσ levels in the cell body, and so it remains possible that after nerve injury PTPσ protein is upregulated on the axon and growth cones of the regenerating cells. Indeed, the related RPTP LAR enhances neurite initiation and is increased after nerve injury and factors that increase the rate of recovery are associated with reduced LAR levels (Lorber et al., 2004; Lorber et al., 2005).

Several studies have interfered with PTPσ-ligand interactions. In the intact retinotectal projection overexpression of a VSV-tagged PTPσ extracellular domain protein produced significant pathfinding errors (Rashid-Doubell et al., 2002). This protein was expressed in a mosaic fashion in the tectum where it bound to the tectal outer limiting basement membrane, which forms the substrate for ingrowing RGC axons, potentially blocking PTPσ ligands that are located in this region (Haj et al., 1999; Mey and Thanos, 2000). The abnormal axons predominantly fail to reach their correct location suggesting that the PTPσ-ligand interaction supports RGC axon extension. Similarly, perturbing the interaction of PTPσ with basement membrane heparin sulphate ligands reduces neurite outgrowth in culture (Ledig et al., 1999a; Mueller et al., 2000). In contrast, it has been suggested that PTPδ-ligand interactions might reduce neurite outgrowth (Johnson et al., 2001). However, it is unclear what the effect on PTP phosphatase activity as distinct from "PTP function" is in each case. The DLAR ligands, syndecan (Sdc) and dally-like protein (Dlp) act antagonistically to regulate the activity of DLAR in synaptogenesis but not axon guidance (Fox and Zinn, 2005; Rawson et al., 2005). However, a unifying hypothesis does not require two kinds of antagonistic ligand-mediated effect but rather one ligand, whose binding alters PTPσ activity and others that compete with the functional ligand for a shared PTP binding site but which have no effect on PTPσ activity themselves. Given the high basal activity of PTPs and the dominance of the inhibitory Dlp over Sdc, it is most likely that PTPσ catalytic activity is inhibited by binding of specific ligands and that other ligands

compete for PTPσ binding but do not affect PTP activity. It is unclear what effect the PTPσ ligands, agrin, collagen 18 or nucleolin have on PTPσ catalytic activity (Aricescu et al., 2002; Sajnani-Perez et al., 2003; Alete et al., 2006). However, overexpression of catalytically inactive PTPσ has a similar phenotype to perturbation of the basement membrane heparin sulphate ligand-PTPσ interaction (Johnson et al., 2001; Rashid-Doubell et al., 2002). Therefore, it can be suggested that this ligand inhibits PTPσ catalytic activity.

In addition to acting as a receptor for a variety of extracellular cues, PTPσ may also have some ligand-like activity. This might explain why some type II RPTP functions require the presence and activity of the tyrosine phosphatase domains whereas others only require extracellular domain expression (Maurel-Zaffran et al., 2001; Marlo and Desai, 2006). Studies on the *Drosophila* type IIa RPTP, DLAR, have revealed that it functions in both a cell autonomous and non-cell autonomous manner during axon guidance, actin polarisation and *oskar* localisation (Maurel-Zaffran et al., 2001; Bateman et al., 2001; Frydman and Spradling, 2001; Krueger et al., 2003). Several approaches have suggested that this type of signalling has been retained by vertebrate type II RPTPs (Wang and Bixby, 1999; Drosopoulos et al., 1999; Sun et al., 2000b). Normal axon regeneration after nerve injury requires PTPσ expression by the nerve and the nerve sheath down which it regenerates. However, regeneration occurs faster in the absence of PTPσ (McLean et al., 2002). Although PTPσ inhibits axon regeneration, the ectodomains of type II RPTPs support neurite outgrowth (Wang and Bixby, 1999; Drosopoulos et al., 1999; Sun et al., 2000b; Yang et al., 2003; Sajnani et al., 2005; Yang et al., 2005). It is unclear whether the non-cell autonomous effects of type IIa RPTPs reflect authentic reverse or bidirectional signalling as described for the Eph/Ephrin and the PTPζ-contactin interactions, or a ligand-like activity of the shed RPTP ectodomain on an as-yet undescribed receptor (Peles et al., 1995; Holland et al., 1996; Mellitzer et al., 2000). Moreover, it has not been determined whether the receptor for the non-cell autonomous effects of PTPσ is an isoform of PTPσ itself as appears to be the case for other type IIa RPTPs (Baker et al., 2000b; Yang et al., 2003; Yang et al., 2005). However, PTPσ -/- axons exhibit path-finding defects that are ameliorated by PTPσ expression in wild type nerve sheaths, suggesting that another receptor may mediate the non-cell autonomous effects of PTPσ in this system.

1.2.3: Interactions of type IIa RPTPs

Finally, a number of studies have attempted to identify the pathways with which PTPσ and related RPTPs interact in order to bring about the cellular functions described

above. A limited number of these studies have looked at PTPσ directly. However, it is likely that inferences can be made based on the findings of studies that examined PTPδ, LAR or invertebrate type IIa RPTPs. To date, only one type IIa RPTP signalling cascade has been described in detail. Binding of the soluble LAR FN5C isoform to LAR on target cells can promote neurite outgrowth (Yang et al., 2003). This is associated with transactivation of the cell surface receptor TrkB. Therefore, the effects of LAR FN5C binding are augmented by neurotrophin administration. TrkB transactivation is likely to account for the subsequent activation of phospholipase-Cγ and phosphoinositide-3-kinase, which in turn activate protein kinase C and B, respectively. Focal adhesion kinase (FAK) is also activated, coupling LAR-induced neurite outgrowth to the regulation of cell-cell adhesion by integrins. Interestingly, FAK may be directly activated by the LAR-interacting protein, TRIO (Medley et al., 2003). Src is also activated in LAR FN5C-induced neurite outgrowth potentially by direct dephosphorylation of the inhibitory Y509 tyrosine residue by LAR (Tsujikawa et al., 2002). Finally, LAR FN5C binding is likely to induce transcriptional changes since mitogen activated protein (MAP) kinase and cyclic AMP response element binding protein (CREB) pathways are activated by LAR FN5C binding (Krauss, 2001; Yang et al., 2005). Lower resolution studies have revealed that type IIa RPTPs interact with a wide variety of cell signalling systems. These relationships include interactions with canonical axon guidance molecules, cytoplasmic and receptor tyrosine kinases, and with the cytoskeleton through interactions with Rac-family GTPases via proteins such as TRIO and through direct interactions with cell adhesion molecules. Finally, a novel family of proteins, the liprins, were first characterised through their interaction with type IIa RPTPs.

1.2.3.1: Classical axon guidance molecules

Whilst PTPσ has not been directly linked to any of the classical axon guidance molecules, the related RPTPs CLR-1, PTP-3, DPTP10D DPTP69D and PTPRO have been linked to the ephrin, netrin and slit axon guidance pathways (Tessier-Lavigne and Goodman, 1996; Sun et al., 2000a; Yu and Bargmann, 2001; Harrington et al., 2002; Dickson, 2002; Chin-Sang et al., 2002c; Huber et al., 2003; Chang et al., 2004; Chilton, 2006; Shintani et al., 2006). PTPRO may function in axon guidance through competition with PTPσ and PTPδ, which potentially places these RPTPs in an Eph related pathway (Stepanek et al., 2005). Although RPTPs have not yet been shown to interact with semaphorins in axon guidance, CD45 interacts with the immune cell semaphorin, CD100 (Elhabazi et al., 2003). It has not yet proven possible to delineate the level at which PTPs

act in these pathways, although they may function to regulate the sensitivity of pathways to ligand binding or to allow the specific ativation of some receptor stimulated behaviours and not others (Haugh et al., 2004; Chang et al., 2004; Shintani et al., 2006). Alternatively, in some systems, RPTP regulated pathways act in parallel to these well described pathways (Dalpe et al., 2005).

1.2.3.2: Receptor and cytoplasmic tyrosine kinase pathways

RPTPs modulate growth factor signalling in response to several stimuli (Kulas et al., 1995; Li et al., 1996; Kulas et al., 1996; Phung et al., 1997; Mooney et al., 1997; Kokel et al., 1998; Suarez et al., 1999; Wang et al., 2000; Graness et al., 2000; Qiao et al., 2001; Xu et al., 2005; Machide et al., 2006). It has been proposed that a major function of PTPs might be to set the threshold and gain of phosphotyrosine signalling, controlling the response of signalling pathways to activation by different amounts of ligand or in different cellular environments (Weiss et al., 1997; Weng et al., 1998; Weng et al., 1999; Tisi et al., 2000; Lei et al., 2002). There is evidence in the case of LAR, that excessive PTP-mediated inhibition of receptor signalling may be of clinical relevance, causing insulin resistance (Ahmad et al., 1995; Ahmad et al., 1997; Ahmad and Goldstein, 1997; Ren et al., 1998; Cheung et al., 2000; Zabolotny et al., 2001; Tagami et al., 2002; Frederiksen et al., 2003; Miscio et al., 2004; Mander et al., 2005). PTPs may mediate these effects in a number of ways including dephosphorylation of the RTK tyrosine residues required for full activation or recruitment of downstream effectors or by dephophosphorylation of key components of signalling cascades downstream of receptor activation (Wang et al., 2000; Cheung et al., 2000; Zabolotny et al., 2001; Mander et al., 2005; Machide et al., 2006). Finally, RPTPs can have effects on the dimerisation of receptor tyrosine kinases presumably by forming heterodimers with kinase proteins (Qiao et al., 2001).

Many ligand regulated cell-signalling pathways do not contain a receptor tyrosine kinase but mediate their effects through activation of cytoplasmic tyrosine kinases such as Src, Abl, Lck and Fyn. The interaction of cytoplasmic tyrosine kinases with RPTPs including RPTPα and CD45 is well described (Zheng et al., 1992; den Hertog et al., 1993; Bhandari et al., 1998; Ponniah et al., 1999; Su et al., 1999; Zheng et al., 2000; Brandt et al., 2003; Pallen, 2003; Shrivastava et al., 2004; Maksumova et al., 2005). So far, there is less evidence for direct interactions of vertebrate type IIa RPTPs with cytoplasmic tyrosine kinases (Tsujikawa et al., 2002). However, the Ableson (Abl) cytoplasmic tyrosine kinase and DLAR counteract each other in *Drosophila* motor neuron axon guidance possibly

through the regulation of the F-actin assembly dynamics (Kaufmann et al., 1998; Wills et al., 1999a; Wills et al., 1999b; Witke, 2004). Ena regulates actin polymerisation, promoting lamellipodial protrusion and is potentially both a LAR and Abl substrate, it is tempting to speculate that this is mediated by Abl and DLAR having directly opposing effects at a single site (Comer et al., 1998; Wills et al., 1999a; Biswas et al., 2002; Sutherland and Way, 2002; Bear et al., 2002; Krause et al., 2003; Gitai et al., 2003). The functional significance of Ena phosphorylation is uncertain, although it may affect its interactions with other proteins resulting in reduced Ena activity and increased actin branching (Kaufmann et al., 1998; Comer et al., 1998; Sutherland and Way, 2002; Krause et al., 2003). In some systems, it appears as though DPTP69D may play a similar and DPTP99A an opposing role (Desai et al., 1997a; Wills et al., 1999a).

1.2.3.3: TRIO proteins and the Ras-related small GTPase family

Changes in the actin cytoskeleton are an integral part of neurite outgrowth and axon guidance. Actin-based cell motility is regulated in part by small Ras-related GTPases, including Rac, RhoA and cdc42 (Hall, 1993; Nobes and Hall, 1995; Luo et al., 1997; Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005; Hall, 2005). Ras family GTPases have intrinsic GTPase activity and cycling between the active GTP-bound form and the inactive GDP bound form is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Quilliam et al., 1995; Rossman et al., 2005). A link between type IIa RPTPs and Ras-family GTPases is suggested by the similar phenotypes that are seen following disruption of the actin cytoskeleton and in DLAR or Ras-family GTPase mutants (Kaufmann et al., 1998). In addition, DLAR mutations interact with Rac1 mutations and PTPδ interacts with the actin-binding protein, MIM-B (Bateman et al., 2001; Woodings et al., 2003). TRIO was first identified as a LAR D2-interacting protein and is the archetypal member of a large protein family that includes TRIO-homologues in *Drosophila* (DTRIO) and *C.elegans* (UNC-73) as well as paralogues including the kalirins (Debant et al., 1996; Alam et al., 1996; Alam et al., 1997; Steven et al., 1998; Kawai et al., 1999; Ratovitski et al., 1999; Bateman et al., 2000; Awasaki et al., 2000; Liebl et al., 2000; Newsome et al., 2000b; Bateman and Van Vactor, 2001; Alam et al., 2001; Daggett et al., 2004; Tse et al., 2005).

TRIO contains four spectrin-like repeats, two guanine nucleotide exchange factor (GEF) domains (Dbl-homology (Diffuse B-cell lymphoma oncogene product; DH)-type), two SH3 domains, two Ig domains and a serine-threonine kinase domains (Debant et al.,

1996). UNC-73 and DTRIO differ in structure from TRIO, most significantly lacking the LAR interacting domain although the significance of these differences is unclear as DLAR and DTRIO still interact at a genetic level (Debant et al., 1996; Bateman et al., 2000; Awasaki et al., 2000; Liebl et al., 2000; Newsome et al., 2000b; Maurel-Zaffran et al., 2001). The function of the non-GEF domains remains unclear although the Ig domains may function to bind and position GTPases and the serine-threonine kinase domain may autophosphorylate TRIO, which may regulate TRIO function (Kawai et al., 1999; Medley et al., 2000; Penzes et al., 2003). In addition, TRIO has the ability to activate downstream signalling proteins directly without a requirement for GEF activity (Bellanger et al., 2000; Seipel et al., 2001). Alternatively spliced isoforms may also play a role in the regulation of TRIO family members, as exemplified by isoforms of kalirin (Mains et al., 1999; Penzes et al., 2001; Estrach et al., 2002; May et al., 2002; Xin et al., 2004; Chakrabarti et al., 2005; Rabiner et al., 2005; Steven et al., 2005; McPherson et al., 2005; Portales-Casamar et al., 2006).

DH domains such as those found in TRIO preferentially bind specific members of the Ras GTPase superfamily. In general, Rac1 activation leads to lamellipodia formation and RhoA activation to growth cone retraction (Luo et al., 1997). It is not clear whether TRIO function requires the activity of both GEF domains (Steven et al., 1998; Bateman et al., 2000; Awasaki et al., 2000; Newsome et al., 2000b; Spencer et al., 2001; Hakeda-Suzuki et al., 2002). However, both the isolated amino-terminal TRIO GEF domain (GEF1) and GEF2 are catalytically active, GEF1 activating the Mig2 or Mig-2-like (Mtl) GTPase and RhoG/Rac1 with subsequent RhoG-dependent activation of Rac1 and cdc42hs whereas GEF2 activates RhoA (Debant et al., 1996; Zipkin et al., 1997; Bellanger et al., 1998; Gauthier-Rouviere et al., 1998; Blangy et al., 2000; Honigberg and Kenyon, 2000; Newsome et al., 2000b; Wu et al., 2002; Kishore and Sundaram, 2002; Struckhoff and Lundquist, 2003; Dalpe et al., 2005). Thus, TRIO is ideally situated to function as a key regulator of GTPase activity in specific developmental processes including a subset of axon guidance decisions (Wu et al., 2002).

The function of TRIO proteins is unknown. Their multi-domain structure suggests a role as scaffolding proteins. This suggestion is supported by the severity and widespread nature of the defects seen in TRIO mutants, particularly UNC-73 mutants, which suggest that TRIO is not part of a single pathway but rather a focal point linking several pathways to the Ras GTPase activation (Bateman and Van Vactor, 2001). These might include PAK, Abl, Ena, reactive oxygen species (ROS) production and signalling and the netrin, robo,

integrin and semaphorin signalling pathways (Bashaw and Goodman, 1999; Wills et al., 1999b; Liebl et al., 2000; Bashaw et al., 2000; Newsome et al., 2000b; Sigal et al., 2003; deBakker et al., 2004; Forsthoefel et al., 2005; Schiller et al., 2005; Mizrahi et al., 2005; Henson, 2005; Lee et al., 2005; Dalpe et al., 2005).

Animals lacking TRIO have significant abnormalities in the development of the nervous system and also in secondary myogenesis, a phenotype that bears comparison with that of PTPσ/PTPδ double mutants (O'Brien et al., 2000; Uetani et al., 2006). Mutations in the *C.elegans* TRIO homologue, UNC-73, produce an uncoordinated phenotype and exhibit a number of abnormalities in axon guidance (Hedgecock et al., 1987; Desai et al., 1988; Siddiqui, 1990; Siddiqui and Culotti, 1991; McIntire et al., 1992; Wightman et al., 1997; Morck et al., 2003), cell migration (Hedgecock et al., 1987; Desai et al., 1988; Chen et al., 1997) and asymmetric cell division (Way et al., 1992), this last possibly through an effect on cell polarisation (Run et al., 1996; Honigberg and Kenyon, 2000; Kishore and Sundaram, 2002). DTRIO mutations result in defects in wing development, dorsal closure and axon guidance abnormalities (Bateman et al., 2000; Awasaki et al., 2000; Liebl et al., 2000; Newsome et al., 2000b). The role of DTRIO in the adult mushroom body organs may be related to the adult expression of PTPσ and PTPδ in the adult vertebrate hippocampus and cerebellum (1.2.1.2). Finally, although DTRIO has only been shown to interact with DLAR, the photoreceptor phenotype of the DTRIO mutant resembles that of DPTP69D mutants, suggesting that DTRIO may interact with other type IIa RPTPs (Garrity et al., 1999).

1.2.3.4: Cell adhesion molecules

The role of PTPs in cell-cell and cell-matrix adhesion has recently been reviewed (Burridge et al., 2006; Sallee et al., 2006). In particular, type II RPTPs interact with cell adhesion molecules including integrins, cadherins and catenins (Matsunaga et al., 1988; Bixby and Zhang, 1990; Riehl et al., 1996; Stone and Sakaguchi, 1996; Aicher et al., 1997; Bateman et al., 2001). DLAR and the β-catenin/N-cadherin system cooperate in axon guidance, opposing Abl function (Iwai et al., 1997; Loureiro and Peifer, 1998; Wills et al., 1999a; Maurel-Zaffran et al., 2001; Clandinin et al., 2001; Lee et al., 2001). However, type IIa RPTP-cadherin interactions are poorly understood. In contrast, type IIb RPTPs regulate signalling pathways downstream of cadherins that include PKCδ and cdc42 (Brady-Kalnay et al., 1998; Burden-Gulley and Brady-Kalnay, 1999; Mourton et al., 2001; Rosdahl et al., 2002; Rosdahl et al., 2003; Ensslen and Brady-Kalnay, 2004). PTPμ controls

cdc42 via IQGAP1, which may play a similar role in PTPμ function to that occupied by the Ras-family GEF, TRIO, in type IIa RPTP function (Phillips-Mason et al., 2006). In addition, RPTP/cadherin interactions affect RPTP function, probably through effects on the sub-cellular distribution or expression level of β-catenin/Armadillo, which is a potential type II RPTP substrate (Brady-Kalnay et al., 1995; Kypta et al., 1996; Fuchs et al., 1996; Burden-Gulley and Brady-Kalnay, 1999; Muller et al., 1999; Symons et al., 2002; Chattopadhyay et al., 2003; Lilien and Balsamo, 2005; Brembeck et al., 2006). Type II RPTPs may also affect the nuclear functions of β-catenin (Anders et al., 2006).

1.2.3.5: LAR-interacting-protein.1 (LIP.1)-related-proteins (Liprins)

The Liprin family consists of α- and β-liprins, which are characterised by amino-terminal coiled-coil, which mediate protein-protein interactions including subtype-specific homodimerisation (i.e. α-liprin:α-liprin/β-liprin:β-liprin), and carboxyl-terminal sterile α motif (SAM) domains, which mediate subtype specific heterodimerisation (i,e, α-liprin/β-liprin) and the α-liprin-type IIA RPTP (LAR) interaction (Serra-Pages et al., 1995; Pulido et al., 1995b; Serra-Pages et al., 1998; Wyszynski et al., 2002; Kriajevska et al., 2002; Schoch et al., 2002; Shin et al., 2003; Ko et al., 2003b; Hofmeyer et al., 2006). Dominant negative liprin splice variants affect liprin interactions and liprin activity may be regulated by ubiquitination or phosphorylation (Serra-Pages et al., 1995; Wyszynski et al., 2002; Kriajevska et al., 2002; Im et al., 2003; van Roessel et al., 2004; Teng and Tang, 2005; Hofmeyer et al., 2006). Phosphorylation is particularly intriguing because liprin phosphorylation affects its interaction with type IIa RPTPs and the major source of liprin phosphorylation might be an intrinsic serine kinase activity of the liprin proteins themselves (Serra-Pages et al., 2005). The function and regulation of liprins remains poorly understood although they appear to act as scaffolding or adaptor proteins (Wyszynski et al., 2002; Schoch et al., 2002; Wang et al., 2002b; Shin et al., 2003; Kim et al., 2003; Ko et al., 2003a; Ko et al., 2003b; Olsen et al., 2005). Moreover, the LAR/liprin interaction represents only a small part of liprin function, which includes roles in axon guidance, synaptic zone assembly, and other aspects of neurotransmission (Desai et al., 1996; Krueger et al., 1996; Kaufmann et al., 1998; Garrity et al., 1999; Zhen and Jin, 1999; Wills et al., 1999a; Newsome et al., 2000a; Maurel-Zaffran et al., 2001; Clandinin et al., 2001; Bateman et al., 2001; Frydman and Spradling, 2001; Lee et al., 2001; Wyszynski et al., 2002; Kaufmann et al., 2002; Ackley et al., 2005; Choe et al., 2006; Hofmeyer et al., 2006). Therefore, the significance of the LAR/liprin interaction is unclear (Baran and Jin, 2002). It was suggested that liprins might function to localise LAR proteins however, liprins, LAR,

and potential LAR ligands function cooperatively to determine their mutual localisation (Serra-Pages et al., 1995; Serra-Pages et al., 1998; Ackley et al., 2003; Ko et al., 2003a; Ko et al., 2003b; Ackley et al., 2005; Hofmeyer et al., 2006). A single explanation reconciling the many effects of liprin proteins might be found in the involvement of liprins in anterograde transport. It has been suggested that liprins might act as kinesin cargo receptors for a number of protein complexes (Ahmari et al., 2000; Shin et al., 2003; Miller et al., 2005; Olsen et al., 2005). Such cargoes are not passively freighted but synergise with their cargo receptor and other cargoes to derive an appropriate sub-cellular localisation, as is seen in the LAR-liprin system (Setou et al., 2002; Dunah et al., 2005).

1.3: RPTP regulation

It was apparent from the discovery of tyrosine kinases that a system must exist to dephosphorylate their substrates. However for technical and philosophical reasons it took almost ten years after the identification of the first tyrosine kinase before the first tyrosine phosphatase was identified and sequenced (Hunter and Sefton, 1980; Tonks et al., 1988; Tonks and Neel, 2001). With the exception of catalytically inactive tyrosine phosphatases (Wishart et al., 1995; Wishart and Dixon, 1998) and those PTPs that act on substrates other than tyrosine phosphate (Martins and Shuman, 2002; Ohta et al., 2003; Laporte et al., 2003; Koreth and van den Heuvel S., 2005), the principle function of PTPs may be assumed to be to counteract the activity of tyrosine kinases. It is clear that tyrosine dephosphorylation can have both positive and negative effects on cell signalling, dependent on the effects of individual tyrosine phosphorylation events on protein function, and that many tyrosine phosphatases are well equipped to function in signalling cascades independently of tyrosine kinases (Marx, 1991).

The regulation of tyrosine phosphatase activity remains unclear and this thesis includes experiments designed to improve our understanding of this area. In principle, it must lie somewhere between two extremes. Firstly, a small number of constitutively active, broad specificity enzymes might act to mop up any unprotected tyrosine phosphate residues, thereby rapidly terminating any tyrosine kinase activity that is not prolonged or supported in some way. Such a model would be suggested by the high basal activity level of tyrosine phosphatases (Streuli et al., 1989; Guan et al., 1990), their broad substrate specificity in vitro (Zhang, 2002), the ability of related PTPs to compensate for the disruption of individual PTPs (Sun et al., 2000a; Schindelholz et al., 2001; Stepanek et al., 2005; Uetani et al., 2006) and the extremely low level of tyrosine phosphate seen in cells

(Hunter and Sefton, 1980). Whilst enzymes that require activation (i.e. tyrosine kinases) can be maintained in the same cellular environment as their substrates, it is likely that highly active enzymes (i.e. tyrosine phosphatases) must be kept separate from their substrates until their activity is needed. Therefore tyrosine phosphatase activity might require regulatory factors to colocalise enzyme and substrate as is seen with the serine/threonine phosphatases (Faux and Scott, 1996a; Faux and Scott, 1996b). Alternatively, a larger number of enzymes might be activated to dephosphorylate individual tyrosine phosphate residues in a specific manner. This would be similar to the regulation of tyrosine kinases. Indeed, the number of tyrosine phosphatases (107) in the human genome is similar to that of tyrosine kinases(90), suggesting that there may be a phosphatase for every kinase although the substrate profile of individual tyrosine phosphatases and kinases do not appear to match exactly (Manning et al., 2002; Alonso et al., 2004). Such a model would require tyrosine phosphatases to exhibit significant substrate specificity, at least *in vivo*. Despite their lack of specificity *in vitro*, PTPs do appear to exhibit some degree of substrate specificity in living cells (Sarmiento et al., 2000; Zhang, 2002; Vetter and Zhang, 2002). It may be that the various classes of tyrosine phosphatase are regulated and function in different ways.

The function of RPTPs is made more complex than that of their cytoplasmic cousins by their ability to directly sense and respond to extracellular cues. As described elsewhere, RPTPs may function in a number of ways (Johnson and Van Vactor, 2003). Some RPTPs may not possess functional ligands in the classical sense – soluble or transmembrane proteins that bind to the receptor and alter its activity. Instead, the RPTP extracellular domains may function to control their inclusion in higher order protein complexes. Alternatively, RPTPs may have cognate ligands that affect tyrosine phosphatase activity (increase or decrease). It may not be reasonable to propose a single regulatory schema for all RPTPs.

The functional activity of RPTPs is likely to be regulated in two ways: firstly, at the level of the specific activity of the tyrosine phosphatase enzyme and secondly by altering the relative localisation of RPTPs and their substrates. Although the regulation of RPTPs is an area of active research, several modes of regulation may be distinguished as being important. It is likely that many RPTPs recognise extracellular ligands, which may affect RPTP activity or function. In addition, the activity of many proteins is affected by regulatory mechanisms including oligomerisation, the enzyme-catalysed attachment of chemical groups to specific amino acid side chains of the RPTP peptide backbone (Post-

translational modification) and post-translational cleavage. Finally, it has recently become clear that the cysteine-based catalytic mechanism of RPTPs makes them peculiarly susceptible to the effects of reactive oxygen species (ROS).

1.3.1: Regulation of PTPs by subcellular localisation

The role of localisation in PTP regulation is an emerging field. However, there is evidence that the regulated colocalisation of substrate and enzyme may play an equally important role in PTP as in serine-threonine phosphatase regulation. Numerous PTPs exhibit distinct patterns of subcellular distribution, which are brought about by a diversity of targeting mechanisms, and in some cases PTP localisation is regulated such that PTPs shuttle between different sites in response to stimuli (Frangioni et al., 1992; Flores et al., 1994; Tillmann et al., 1994; Lorenzen et al., 1995; Wang et al., 1996; Cossette et al., 1996; Kamatkar et al., 1996; Aicher et al., 1997; Jena et al., 1997; Tiganis et al., 1997; Tenev et al., 2000; Gjorloff-Wingren et al., 2000; Xu et al., 2001; Craggs and Kellie, 2001; Uchida et al., 2002; Yang et al., 2002; Wang et al., 2002a; Bompard et al., 2003; Herrmann et al., 2003; Seifert et al., 2003; Ianzano et al., 2004; Chirivi et al., 2004; Del Vecchio and Tonks, 2005; van Ham et al., 2005; Gupta and Swarup, 2006; Hernandez et al., 2006). However, specific effects of PTP localisation have so far only been elucidated in a minority of cases. Localisation of the *Yersinia pestis* (Plague) tyrosine phosphatase is an important aspect of the pathogenesis of this disease (Persson et al., 1999). In addition, type II RPTPs generally localise to areas of cell-cell contact, where they interact with components of the actin cytoskeleton or cell-cell adhesions. This area will be covered below when the regulation of RPTPs by post-translational cleavage and the interaction of RPTPs with other proteins are considered (1.2.3.4; 1.3.5). The type IV RPTPs, PTPα and PTPε, are regulated by post-translational cleavage and alternative splicing, which may also be mediated by the differential localisation of the cleaved or alternatively spliced proteins (Gil-Henn et al., 2000; Gil-Henn et al., 2001; Andersen et al., 2001a; Kraut et al., 2002). Finally, one of the most exciting areas of research on RPTP localisation is in the effects of localisation on the role of CD45 in T Cell receptor (TCR) signalling.

The archetypal RPTP, CD45, plays a key role in immune cell signalling. Up to 5-10% of the surface of lymphocytes is coated in CD45 molecules and both T and B cells deficient in CD45 exhibit abnormalities in signalling downstream of their respective antigen receptors. The defect in T cell signalling is the more pronounced and the role of CD45 in TCR signalling is more clearly understood than its role in B cell signalling

(Alexander, 2000). The major substrates of CD45 are *Src*-family tyrosine kinases downstream of the TCR. The regulation of these tyrosine kinases by CD45 is complex as CD45 has both positive (through dephosphorylation of the C-terminal inhibitory tyrosine) and negative (through dephosphorylation of the autophosphorylation site) effects on tyrosine kinase activity (Thomas and Brown, 1999; Baker et al., 2000a). TCR signalling induces the formation of an immune synapse at the T Cell/antigen presenting cell junction (Bromley et al., 2001). Following T cell receptor activation, lipid rafts migrate to the synapse concentrating the components of the TCR signalling system (Montixi et al., 1998; Viola et al., 1999; Kabouridis, 2006). Lipid rafts are sphingolipid- and cholesterol-rich microdomains of the cell membrane to which a number of signalling proteins are specifically targeted and which play an important role in physiological and pathological processes including axon guidance (Dykstra et al., 2003; Edidin, 2003; Simons and Vaz, 2004; Lagerholm et al., 2005; Kamiguchi, 2006). Interestingly, *Src* tyrosine kinases and other components of T cell receptor signalling cascade segregate to lipid rafts whereas CD45 is usually excluded from these regions of the membrane (Rodgers and Rose, 1996; Xavier et al., 1998). However, a proportion of CD45 molecules move between lipid rafts and the remainder of the cell membrane (Johnson et al., 2000; Edmonds and Ostergaard, 2002; Freiberg et al., 2002; Zhang et al., 2005). A model has been suggested where low levels of raft-associated CD45 negatively regulate basal levels of TCR signalling, possibly preventing the effects of inappropriate TCR activation. In contrast, CD45 excluded from lipid rafts plays a positive role in downstream signalling through ERK kinases potentiating the effects of appropriate TCR activation (Miceli et al., 2001; Hermiston et al., 2003). Preliminary data suggests that PTPσ may also localise to lipid rafts (A. Stoker, unpublished data).

1.3.2: Regulation of RPTPs by ligand binding

The structure of many RPTP extracellular domains immediately suggests that these proteins might act as transmembrane receptors for extracellular ligands, which might regulate RPTP tyrosine phosphatase activity (Charbonneau et al., 1988). Epidermal growth factor (EGF) negatively regulates the activity of an EGF receptor extracellular domain-CD45 phosphatase domain chimera, proving that in principle, ligands have the potential to regulate RPTP activity and that ligands are likely to inhibit PTP catalytic activity (Desai et al., 1993). However, the *in vivo* function of CD45 is rescued by a chimaera lacking the endogenous CD45 extracellular domain (Hovis et al., 1993). Thus, the function of RPTP extracellular domains remains unclear. It must be noted however, that CD45 is expressed at extremely high levels by nucleated blood cells, covering up to 10% of the cell surface (Cited

in Thomas, 1989). Therefore, CD45 regulation may not need the influence of the extracellular domain to regulate CD45 localisation or protein-protein interactions. Furthermore, the number of proteins known to interact with RPTP extracellular domains is small and even fewer have been shown to have an effect on RPTP function or activity. The lack of an assayable RPTP function or identified substrates for many RPTPs has made it extremely difficult to evaluate these putative ligands. Indeed for the majority of the RPTP family, "it is mainly an article of faith that such physiologically relevant ligands really exist" (Bixby, 2001). Several different types of ligand-RPTP interactions will be discussed below.

Firstly, most reminiscent of a canonical ligand-receptor interaction, several RPTPs including PTPσ bind proteins expressed on other cells or in the extracellular environment (in *trans*). Extracellular domain ligands have been reported for PTPσ, PTPζ (PTPRZ), CD45 and DEP1 (see below for references). In addition, a putative IA2 ligand has been located in pancreatic islets however, it has not been characterised further (Chiang and Flanagan, 1996). At least two kinds of candidate PTPσ ligands have been identified, a heparan sulphate proteoglycan (HSPG) in basement membranes and the protein, nucleolin, in developing muscle (Aricescu et al., 2002; Sajnani-Perez et al., 2003; Alete et al., 2006). In addition, PTPσ has been identified as one of a number of candidate targets for a component of Black Widow venom (Krasnoperov et al., 2002). There is no appropriate functional PTPσ assay currently available to determine the effect of these candidate ligands on PTPσ. Therefore, it remains to be determined whether these two potential PTPσ ligands represent authentic ligands, affecting PTPσ function or activity or whether they play a different role, for example as a co-receptor as is seen for HSPGs in other signalling systems (Lin, 2004). The *Drosophila* type IIa RPTP, DLAR, also recognises HSPGs and vertebrate LAR recognition of the laminin-nidogen complex promotes neurite outgrowth, suggesting that the recognition of extracellular matrix components including HSPGs may be a common feature of this RPTP family (O'Grady et al., 1998; Fox and Zinn, 2005; Johnson et al., 2006). The effects of the laminin-nidogen complex on LAR function are also unknown. However, the closely related *Drosophila* HSPGs, *Syndecan* and *Dally-like Protein,* appear to either increase or decrease DLAR activity respectively (Fox and Zinn, 2005; Johnson et al., 2006). Similarly, an undefined extracellular matrix ligand has been reported to increase DEP1 (PTPRJ) phosphatase activity (Sorby et al., 2001). It may be possible to predict the effect of PTPσ ligands from studies on other RPTPs.

In contrast to the situation for PTPσ, both substrates and ligands have been identified for PTPα, PTPζ and CD45. PTPα is inhibited by an undefined ligand in serum

(Blanchetot and den Hertog, 2000a). However, the effect of ligand binding on PTPα dimerisation was not determined. PTPζ ligands fall into two distinct categories. A number of Fibronectin-type 3 domain-containing Ig super-family cell adhesion molecules and extracellular matrix components bind to PTPζ through interactions that require the N-linked glycosylation of PTPζ but not its chondroitin sulphate sidechains, which may in fact inhibit binding of FN3 domain-containing ligands (Barnea et al., 1994; Milev et al., 1994; Grumet et al., 1994; Milev et al., 1995; Peles et al., 1995; Milev et al., 1996; Milev et al., 1997; Milev et al., 1998a; Revest et al., 1999; Garwood et al., 2003). The PTPζ interaction with these proteins may initiate bidirectional signalling (Peles et al., 1995; Revest et al., 1999). However, the effect of these interactions on PTPζ function is not known. In contrast, the interaction of PTPζ with heparin-binding proteins such as pleiotrophin, midkine, Fibroblast Growth Factor-2 or amphoterin may require the chondroitin sulphate sidechains (Maeda et al., 1996; Maeda and Noda, 1998; Milev et al., 1998a; Milev et al., 1998b; Maeda et al., 1999; Maeda et al., 2003; Sakaguchi et al., 2003). Moreover, pleiotrophin-PTPζ binding inhibits the intrinsic tyrosine phosphatase activity of this RPTP towards its substrates including the *Src*-family tyrosine kinase, *Fyn*, and the key developmental and cell signalling molecule, β-catenin (Meng et al., 2000; Kawachi et al., 2001; Fukada et al., 2005; Pariser et al., 2005a; Pariser et al., 2005b). Significantly, inhibition of PTPζ phosphatase activity by pleiotrophin is associated with PTPζ clustering and a similar effect is seen by systems that artificially dimerise PTPζ in response to ligands (Fukada et al., 2006).

Several lectins including galectin-1 (galectin), are candidate CD45 ligands (Walzel et al., 1999; Rachmilewitz et al., 2003). Galectin exists in an equilibrium between a monomeric and dimeric form *in vivo,* and multiple galectin molecules are able to bind to CD45 in a carbohydrate-dependent fashion (Cho and Cummings, 1995; Symons et al., 2000). However, although treatment of T cells with galectin induces apoptosis (Perillo et al., 1995), this effect is not mediated by the galectin-CD45 interaction but through binding of galectin to other cell membrane proteins including CD3, CD4 CD7 and CD42 (Fajka-Boja et al., 2002). In fact, the galectin-CD45 interaction serves to regulate the susceptibility of T cells to apoptosis induced by galectin treatment rather than to induce apoptosis *per se.* The effects of the galectin-CD45 interaction are complex and depend on the glycosylation status of CD45 (1.3.3). However, the resistance of O-linked glycosylation deficient cells to galectin induced cell death requires CD45 catalytic activity, suggesting that galectin binding to normal cells inhibits CD45 catalytic activity (Nguyen et al., 2001). Moreover in

susceptible cells, galectin treatment results in increased CD45 clustering (Pace et al., 1999), decreased CD45 tyrosine phosphatase activity (Walzel et al., 1999; Fouillit et al., 2000b) and decreased *Lyn* tyrosine kinase activity (a CD45 substrate) (Katagiri et al., 1999; Fouillit et al., 2000a; Fouillit et al., 2000b). In contrast, in cells that are resistant to galectin-induced cell death, there is decreased CD45 clustering and a requirement for higher levels of CD45 phosphatase activity (Nguyen et al., 2001). As galectin is potentially a divalent ligand, an obvious possibility is that galectin treatment causes receptor dimerisation, which is associated with tyrosine phosphatase inhibition as in the PTPζ system. This has been shown for the PP14-CD45 interaction (Rachmilewitz et al., 2003).

Secondly, several RPTPs exhibit homophilic binding. Although PTPσ has not been shown to interact homophilically, the remaining two members of the vertebrate type IIa RPTP family (PTPδ, LAR), all studied type IIb RPTPs (PTPμ, PTPκ) and the related leech type IIa RPTP, HmLAR2, all interact homophilically (Brady-Kalnay et al., 1993; Gebbink et al., 1993b; Brady-Kalnay and Tonks, 1994; Sap et al., 1994; Zondag et al., 1995; Wang and Bixby, 1999; Baker et al., 2000b; Yang et al., 2003; Cismasiu et al., 2004; Yang et al., 2005; Aricescu et al., 2006). It is therefore likely that PTPσ also interacts in this fashion. Although in the case of type IIb RPTPs, these interactions can occur both between PTPμ molecules on opposing cells (in *trans*) and between PTPμ molecules in the same cell membrane (in *cis*)(Cismasiu et al., 2004; Aricescu et al., 2006). As these interactions involve the PTPμ MAM domain, the homophilic interactions exhibited by LAR and PTPδ must utilise an overlapping or distinct mechanism. It is of note that the homophilic interaction of LAR protein requires the presence of a small alternatively spliced exon. Although this exon is conserved in the PTPσ and PTPδ genes, it was not present in the PTPσ isoforms in which homophilic adhesion has been assayed and it is unclear whether it is required for PTPδ mediated homophilic adhesion (O'Grady et al., 1994). Although homophilic interactions between RPTPs have clear functional consequences at the level of cell behaviour (Wang and Bixby, 1999; Yang et al., 2003) it is not yet possible to state what effect they have on the function or activity of the RPTP involved . However, there are suggestions that homophilic interactions between LAR molecules might stimulate LAR tyrosine phosphatase activity (Yang et al., 2005).

Finally, the binding of transmembrane ligands by RPTPs expressed on the same cell (in *cis*) has been reported. However, at the present time this has only been described for PTPα and the function of the interaction is unclear (Zeng et al., 1999). However, there is the intriguing possibility that the ligand, contactin, might function to link PTPα function to

that of the other RPTP that it interacts with, PTPζ. As contactin interacts with one RPTP in *cis* and the other in *trans,* it might function to link RPTP function in neighbouring cells. Such ligands remain potentially undetectable with many of the methods used to identify RPTP ligands and as such represent an interesting future area of research.

1.3.3: Post-translational modification

Proteins may exhibit a number of different post-translational covalent modifications. These include glycosylation and phosphorylation. The regulation of RPTPs by reversible oxidation will be considered separately as unlike the modifications considered here, it is not mediated by the enzyme-catalysed transfer of a chemical group to the RPTP peptide chain but rather the direct effects of a diffusible chemical on the peptide chain itself. Other covalent protein modifications play a role in cell signalling and protein function (e.g. ubiquitylation (Hershko and Ciechanover, 1998; Voges et al., 1999; Welchman et al., 2005), prenylation and palmitoylation (Huang and El Husseini, 2005)), but the role of these modifications in RPTP function remains to be determined.

Many proteins involved in embryonic development exhibit enzyme-catalysed glycosylation, predominantly on asparagine (N-linked) or serine and threonine (O-linked) residues (Vliegenthart and Casset, 1998; Ten Hagen et al., 2003; Haltiwanger and Lowe, 2004; Weerapana and Imperiali, 2006). In general, glycosylation serves to create unique carbohydrate epitopes that participate in ligand-receptor interactions or to alter the general properties of a protein such as its charge, protease sensitivity or solubility (Varki, 1993). The primary sequences of many RPTPs contain potential glycosylation sites. PTPσ is predicted to contain 1 O-linked and 2 N-linked glycosylation sites.These predictions have been empirically verified in a number of proteins (Daum et al., 1991; Yu et al., 1992; Daum et al., 1994; Sorio et al., 1995; Hermel et al., 1999). In most cases, it is unclear what effect glycosylation has on RPTP function. However, RPTP glycosylation has been shown to affect the susceptibility of PTPκ to proteolytic cleavage (1.3.5), which may be associated with the acquisition of metastatic potential by malignant cells (Kim et al., 2006). In addition, the binding of physiological ligands and toxins are independently affected by PTPβ/ζ glycosylation (Milev et al., 1995; Yahiro et al., 2004). The most well understood effect of glycosylation on RPTP function is seen in the prototypical RPTP, CD45. It has long been appreciated that CD45 isoforms exhibit differential glycosylation thanks to the inclusion of exons containing additional glycosylation sites (Jackson and Barclay, 1989; Pulido and Sanchez-Madrid, 1990; Pulido et al., 1994). These differences have significant

implications for the function of CD45 isoforms in thymocyte maturation and activation, T-cell function in HIV-infected cells, leukaemogenesis, antigen-presenting cell function and immune senescence (Deans et al., 1991; Lefebvre et al., 1994; Ohta et al., 1994; Craig et al., 1994; McKenney et al., 1995; Dang et al., 1996; Wood et al., 1998; Bleesing et al., 2003; Garcia et al., 2005). O- and N-linked glycosylation affect the susceptibility of T cells to galectin and both are required for the induction of cell death by galectin treatment (Galvan et al., 2000; Nguyen et al., 2001; Amano et al., 2003). In addition to differing in the number of glycosylation sites, CD45 isoforms differ in their degree of sialylation. Increased CD45 sialylation reduces galectin binding and CD45 clustering, abrogating the inhibitory effect of galectin on CD45 tyrosine phosphatase activity resulting in reduced galectin-induced cell death. A clinical correlate of this may be the reduced CD45 sialylation seen in HIV infected thymocytes, which is associated with increased exposure of O-linked CD45 glycans and increased susceptibility to cell death (Lanteri et al., 2003). It was suggested that the effects of glycosylation and sialylation on CD45 activity might reflect an effect on CD45 dimerisation, which has been shown to inhibit CD45 phosphatase activity (1.3.4) (Xu and Weiss, 2002; Dornan et al., 2002). This effect might be mediated by direct blockade of the binding site for a dimerising ligand, masking of a CD45 dimerisation motif or the affect of the additional sugar residues on the extracellular domain electric charge. The significant effects of glycosylation on CD45 function and the widespread presence of glycosylation in the RPTP family make it possible that this may represent a widespread form of RPTP regulation.

Although many PTPs are regulated by phosphorylation, it is difficult at present to generalise about the role of conserved potential phosphorylation sites or empirically described phosphorylation events across the family (Su et al., 1994; Ostman and Bohmer, 2001). However, two phosphorylation events show that phosphorylation is likely to play a key role in PTP regulation. Firstly, the C-terminal PTPα Tyr789 residue is a binding site for the Grb2 adaptor protein and facilitates dephosphorylation of the inhibitory c-terminal tyrosine of *Src*-family tyrosine kinases via a phosphotyrosine displacement mechanism (den Hertog et al., 1994; Zheng et al., 2000). The role of Tyr789 is modulated by reactive oxygen species (1.3.6) and by Protein Kinase C phosphorylation of PTPα at several serine residues (S180, S204) (Zheng et al., 2002). *Src*-family kinases play important roles in cell function, many PTPs dephosphorylate Src or its close relatives and a C-terminal tyrosine is a common feature of many RPTPs including those of the type IIa family (Roskoski, Jr., 2005). Secondly, as mentioned above PTPα is constitutively phosphorylated upon two

juxtamembrane serine residues (Tracy et al., 1995). Several lines of evidence suggest that phosphorylation of these residues may play an important role in RPTPα function (Stetak et al., 2001; Zheng et al., 2002). Moreover, these residues are contained in an alternatively spliced exon (1.2) in the type IIa RPTP family, implying that phosphorylation of juxtamembrane serine residues may be important across the RPTP family (Zhang and Longo, 1995).

1.3.4: RPTP oligomerisation

Receptor dimerisation is a major feature of the regulation of many transmembrane protein families. Paradigmatically, receptor tyrosine kinases are activated by ligand induced dimerisation, which facilitates the conformational change required to allow subunit transphosphorylation and kinase activation (Schlessinger, 1986; Schlessinger, 1988; Lemmon and Schlessinger, 1994; Schlessinger, 2002). In addition, G-protein coupled receptors (GPCR) form homo- and heterodimers both as an integral part of signal transduction and to allow cross-talk between different GPCR pathways (Milligan, 2004; Milligan et al., 2004; Kubo and Tateyama, 2005; Fotiadis et al., 2006).

Dimerisation is also a feature of RPTP regulation. The transmembrane domains of many RPTPs dimerise in micelles, a common feature of dimerising transmembrane proteins (Chin et al., 2005). Studies support a role for ligand binding in the inhibition of CD45, RPTPα and possibly PTPμ, mediated through formation of a receptor dimer in which the active site of each subunit is occupied by a conserved juxtamembrane wedge from the other subunit (Desai et al., 1993; Bilwes et al., 1996; Weiss and Schlessinger, 1998; Feiken et al., 2000). In addition to ligand binding, there is evidence that other systems may regulate RPTP dimerisation including post translational modification (Xu and Weiss, 2002) and reactive oxygen species (1.3.6). As is seen with receptor tyrosine kinases, the orientation of the dimer is critical for normal signal transduction (Burke et al., 1997; Burke and Stern, 1998; Jiang et al., 1999; Jiang and Hunter, 1999). The inhibitory role of the juxtamembrane wedge appears to be important in *vivo* for CD45 (Majeti et al., 1998; Majeti et al., 2000; Hermiston et al., 2005). However, this dimeric structure may be related to the selection of a specific region of RPTP sequence for crystalisation (Felberg and Johnson, 1998). Indeed, an alternative dimer configuration is seen with the PTPμ D1 domain that is related to crystalisation conditions (Hoffmann et al., 1997; Eswaran et al., 2006). In addition, the juxtamembrane wedge sequence is not present in all RPTPs and the single phosphatase domain crystal structure which first suggested this model is not seen in all

RPTPs (Hoffmann et al., 1997). Furthermore, deletion of juxtamembrane sequences in some RPTPs reduces activity rather than increasing it in accordance with the model proposed by Majeti et al 1998 (Wang and Pallen, 1992; Gebbink et al., 1993a). Indeed, extant crystal structures of tandem phosphatase domains suggest that they may be unable to form the requisite homodimeric structure to inhibit phosphatase activity in this way (Nam et al., 1999; Nam et al., 2005). Despite this, wedge domain peptides successfully inhibit the activity of RPTPs including those that appear to be unable to form the necessary dimeric crystals (Xie et al., 2006). A role for dimerisation in RTP function is supported by other studies although the structure of the dimer has not been determined in all cases (Takeda et al., 1992; Jiang et al., 2000; Blanchetot and den Hertog, 2000a; Tertoolen et al., 2001; Gross et al., 2002; Blanchetot et al., 2002a; Toledano-Katchalski et al., 2003; Takeda et al., 2004; Walchli et al., 2005). In summary, it appears that dimerisation is an important regulatory mechanism in many RPTPs, although the exact mechanism of dimerisation remains unclear.

A number of alternative mechanisms of tyrosine phosphatase dimerisation have been reported, which are unlikely to play a significant role in RPTP regulation (Tabernero et al., 1999; Akerud et al., 2002; Bernado et al., 2003). In addition, several type II RPTPs form homo-oligomeric complexes mediated by both *cis* and *trans* interactions as described above (Brady-Kalnay et al., 1993; Gebbink et al., 1993b; Brady-Kalnay and Tonks, 1994; Sap et al., 1994; Zondag et al., 1995; Wang and Bixby, 1999; Yang et al., 2003; Cismasiu et al., 2004; Yang et al., 2005; Aricescu et al., 2006). Furthermore, heterophilic interactions have been reported between the D1 and D2 tyrosine phosphatase domains of a number of different RPTPs. The specificity of this interaction is affected by the juxtamembrane wedge region, which may represent the physiological function of the wedge region, and is inhibited by an intramolecular interaction between the D1-D2 spacer region and the C-terminal region of D2 (Wallace et al., 1998; Felberg and Johnson, 1998; Feiken et al., 2000; Hayami-Noumi et al., 2000; Felberg and Johnson, 2000; Blanchetot and den Hertog, 2000b; Gross et al., 2002; Blanchetot et al., 2002a; Blanchetot et al., 2002b; Toledano-Katchalski et al., 2003). This spacer/c-terminal interaction may produce a locked conformation of the D2 domain, unable to interact with other PTP domains, that is analogous to the locked conformation of the *Src*-family tyrosine kinases (Xu et al., 1997; Blanchetot et al., 2002a; Hantschel et al., 2003; Nagar et al., 2003; Harrison, 2003). Finally, it has been suggested that reactive oxygen species may play a role in the regulation of RPTP activity through an effect on dimerisation (1.3.6).

1.3.5: Post-translational cleavage

A striking feature of members of the type II RPTP family is that they undergo sequential post-translational cleavage events. Four distinct kinds of type II RPTP cleavage events have been described. The cleavage of signal peptides by signal peptidase during translocation into the endoplasmic reticulum will not be considered here (Paetzel et al., 2002).

Type II RPTPs possess a conserved dibasic recognition site for cleavage by subtilisin-like protein convertases (PC) that is located approximately 100 amino acids from the plasma membrane, in the membrane proximal FN3 domain (Streuli et al., 1992; Jiang et al., 1993; Yan et al., 1993; Serra-Pages et al., 1994; Pulido et al., 1995a; Campan et al., 1996; Cheng et al., 1997). PCs cleave a wide range of peptide substrates, playing an important role in development, normal physiology and in the pathogenesis of infective, degenerative and malignant disease (Seidah et al., 1994; Thomas, 2002; Seidah and Prat, 2002; Rockwell et al., 2002). The PCs exhibit differing subcellular distributions exemplified by that of furin, which cycles between the trans-Golgi network (TGN), endosomal compartments and cell surface with furin cleavage being either a TGN or a post-TGN event (Seidah et al., 1994; Molloy et al., 1994; Vischer and Wagner, 1994; Molloy et al., 1999; Chen et al., 2001; Band et al., 2001; Thomas, 2002; Seidah and Prat, 2002). Specifically, the cleavage of PTPκ is mediated by furin, and that of PTPμ, by PC5 (Campan et al., 1996; Anders et al., 2006). The enzyme(s) responsible for LAR, PTPδ, PTPσ, PTPλ and PTPϱ cleavage remains unidentified. The precise role of PC mediated cleavage in type II RPTP function is unclear. However, as with other PC substrates, cleavage of type II RPTPs at this site is not required for membrane expression and heterodimeric forms containing both cleaved and uncleaved protein have been reported (Streuli et al., 1992; Jung et al., 1993; Serra-Pages et al., 1994; Pulido et al., 1995a; Bush et al., 2001). Indeed, although furin-mediated cleavage of Notch is required for the subsequent release of the Notch intracellular domain by γ-secretase (De Strooper et al., 1999; Weinmaster, 2000), uncleaved Notch is not an artefactual finding. Rather, it regulates a distinct signalling pathway in response to binding of the Notch ligand, Delta (Bush et al., 2001). The regulation of PC-mediated PTPμ cleavage by cell density suggests that cleaved and uncleaved type II RPTPs may similarly have distinct functions (Campan et al., 1996). It is unknown what effect PC-mediated cleavage has on the ligand binding or signalling properties of type IIa RPTPs. However, PC-mediated cleavage is not required for the ectodomain shedding that is associated with the second form of type IIa RPTP cleavage (Serra-Pages et al., 1994).

Secondly, type II RPTPs are cleaved at a site close to the plasma membrane, which is associated with shedding of the extracellular domain portion and in some cases with internalisation of the intracellular domain (Serra-Pages et al., 1994; Aicher et al., 1997). Cleavage at this site is increased in response to cell density and treatment with phorbol ester, calcium ionophores or anti-RPTP antibody and is associated with an increase in specific PTP activity (Aicher et al., 1997; Anders et al., 2006). It can be hypothesised that this increase may reflect the removal of an inhibitory dimeric structure forced upon the intact protein by an interaction with an extracellular ligand. Many transmembrane proteins including Notch are subject to cleavage by members of the matrix metalloprotease or "a distintegrin and metalloprotease" (ADAM) family (Brou et al., 2000; Lieber et al., 2002; McFarlane, 2003; White, 2003; Seals and Courtneidge, 2003; Huovila et al., 2005; Malemud, 2006). Cleavage is associated with shedding of the extracellular domain with the remaining intracellular portion of the protein being subject to degradation, often via cleavage and release from the membrane by a protease that cleaves within the lipid bilayer (e.g. γ-secretase). However, it is not clear whether cleavage by ADAMs is sufficient for ectodomain shedding or whether additional conformational effects are required. Specifically, ADAM-10 is responsible for cleavage of PTPκ and TACE/ADAM-17 is suggested to mediate LAR cleavage (Ruhe et al., 2006; Anders et al., 2006). The enzymes responsible for the cleavage of other type II RPTPs at this site have not yet been identified.

A third cleavage event has recently been identified for at least some type II RPTPs. Mediated by γ-secretase, it releases the RPTP intracellular domain from the membrane either for transport to the nucleus where it dephosphorylates β-catenin affecting β-catenin regulated transcription or for degradation by the 26S proteasome. Cleavage at this third site requires antecedent cleavage by a sheddase (Anders et al., 2006). Thus in the future, RPTPs may follow tyrosine kinase receptors and be identified as unlikely mediators of nuclear signalling (Ni et al., 2001; Lin et al., 2001).

Finally, the type III RPTPs, PTPα and PTPε are cleaved by calpain within the juxtamembrane region. This releases a soluble intracellular domain fragment with an apparently reduced tyrosine phosphatase activity (Gil-Henn et al., 2000; Gil-Henn et al., 2001). However, the function of this event in type III RPTP function and its relevance to RPTP processing in general remains to be elucidated.

1.3.6: Regulation by reactive oxygen species (ROS)

The effect of certain oxidising agents on the catalytic cysteine of classical tyrosine phosphatases has long been used to inhibit cellular tyrosine phosphatase activity (Huyer et al., 1997; Denu and Tanner, 1998). Recently it has become apparent that hydrogen peroxide (H_2O_2) in particular is not just a pharmacological tool but a major cell signalling molecule in its own right, playing an important role in the signalling induced by growth factor binding to receptor tyrosine kinases (Sundaresan et al., 1995; Bae et al., 1997; Meng et al., 2002). Other signals also have the potential ability to regulate PTP activity by ROS production (Barford, 2004; den Hertog et al., 2005). The major effects of ROS signalling appear to be mediated through redox modification of cysteine residues (Barford, 2004; Rhee, 2006; Biswas et al., 2006). The production of ROS in these signalling cascades is incompletely understood, but probably utilises the same catalytic machinery as the oxidative burst in phagocytic cells through the NADPH oxidase (Nox) complex. Nox generates superoxide, which can be converted into H_2O_2 by superoxide dismutase (Sundaresan et al., 1995; Irani and Goldschmidt-Clermont, 1998; den Hertog et al., 2005; Rhee, 2006).

Tyrosine phosphatases are likely to be the major target of ROS signalling and they specifically regulate distinct aspects of ROS signalling (Hao et al., 2006a); PTP catalysis requires a critical cysteine to be maintained in a reduced state (Zhang and Dixon, 1993; Xu et al., 2002; den Hertog et al., 2005). However, H_2O_2 also has direct effects on protein tyrosine kinases (Giannoni et al., 2005). Oxidation of the PTP catalytic cysteine to sulphenic acid is reversible, whereas further oxidation is less so. It has recently been shown that at least some PTPs are able to protect their catalytic cysteine from irreversible damage by oxidation. The catalytic cysteine may be protected by the formation of homodimeric intramolecular disulphide bonds (Caselli et al., 1998; Lee et al., 1998; Savitsky and Finkel, 2002) or the formation of a ring-form sulfenyl-amide (Salmeen et al., 2003; van Montfort et al., 2003). Although both mechanisms temporarily inhibit phosphatase activity, they are readily reversible to the thiolate ion. It is not known whether RPTPs utilise either of these protective strategies. However, *in vitro* oxidation of PTPα results in the formation of sulphonic acid at the D2 catalytic cysteine but not at the equivalent D1 residue (Persson et al., 2004). This suggests that the D1 cysteine might be less susceptible to oxidation than the D2 cysteine, perhaps because of the tertiary and quaternary structure of PTPα or through utilisation of one of these strategies.

Although H_2O_2 has the potential to cause oxidation of the catalytic cysteine leading to decreased phosphatase activity, it is possible that it in fact has its effects on RPTP function through a quite different mechanism that is just beginning to be elucidated. The membrane distal, D2, domain of PTPα is more susceptible to *in vitro* oxidation than the membrane proximal D1 domain. H_2O_2 treatment inhibits PTPα phosphatase activity, which is associated with the stabilisation of preformed PTPα dimers and the association of PTPα with potential downstream effectors (Blanchetot et al., 2002a; Hao et al., 2006a; Hao et al., 2006b). Interestingly, H_2O_2 treatment also inhibits the intramolecular D2 spacer/c-terminal interaction whilst stimulating an intermolecular D2-D2 interaction. This suggests that the spacer/c-terminal interaction may function to maintain PTPα in an active dimeric state and prevent the intermolecular interaction of D2 domains in the PTPα dimer (Blanchetot et al., 2002a). These effects are associated with a conformational change both in the structure of isolated D2 domains and in the structure of the extracellular region of PTPα dimers. The conformational effect on PTPα D2 domains is conserved in D2 domains from the type IIa RPTP, LAR, and thus is likely to be generally applicable to all two-phosphatase domain RPTPs (Blanchetot et al., 2002b). A similar effect can be produced by altering the axis of dimerisation of constitutively dimeric PTPα (Jiang et al., 1999; van der Wijk et al., 2003). Surprisingly, these effects do not require a functional D1 catalytic cysteine, but appear to be mediated by cysteine residues in D2 (Buist et al., 2000; Blanchetot et al., 2002a; van der Wijk et al., 2003).

In addition, H_2O_2 treatment induces the formation of disulphide crosslinked forms of PTPα (van der Wijk et al., 2004). This effect requires the presence of the D2 catalytic cysteine but it is unclear whether this residue is the site of reciprocal disulphide bond formation between dimer subunits. Disulphide bonded dimers are not formed in dimers consisting of a full-length PTPα molecule and an isolated D2 domain (Blanchetot et al., 2002a). Indeed, the data presented could reflect a requirement for the D2 catalytic domain in sensing ROS and inducing a conformational change in PTPα dimer structure, disulphide bond formation being mediated by other cysteine residues. In the absence of the D2 catalytic cysteine, the conformation of the PTPα dimer might not place the responsible cysteines close enough together for disulphide bond formation. As dimeric receptors commonly exist in equilibrium between "tense" and "relaxed" conformations, even in the absence of ligand stimulation, this might also explain why even in the insensitive D2 catalytic cysteine mutants, a small amount of PTPα would be found in the appropriate conformation for disulphide bond formation.

These results suggest that the major effect of H_2O_2 on RPTP activity might not be mediated through oxidation of the catalytic cysteine as previously assumed but through indirect effects on protein structure via a Redox-sensitive switch incorporating the D2 catalytic cysteine.

1.4: Thesis Aims

The aim of this thesis is to investigate the mechanism of action and function of type IIa RPTPs, in particular PTPσ. A number of approaches were taken to identify the role of receptor oligomerisation in PTPσ function, to evaluate the effects of pharmacological agents including the putative PTPσ ligand, heparin sulphate, on PTPσ function and to identify additional PTPσ–interacting proteins, with a particular emphasis on potential substrates. One aim of this work was to establish an assay that could be used to assess PTPσ activity in response to experimental manipulations. Finally, a major focus of the research of this laboratory is studying the role of RPTPs in chick embryonic axon guidance. Although successful attempts have been made to perturb PTPσ function, these have so far produced subtle phenotypes. In fly models of axon guidance, it has often been necessary to disrupt the function of several PTP family members to produce a phenotype. The chick homologues of PTPσ and PTPδ have been cloned. Therefore, the isolation of the chick homologue of LAR was attempted. This would complete the family of three chick type IIa RPTPs and allow disruption of all three type IIa RPTPs, alone or in combination.

Chapter 2: Materials and Methods

2.1: Materials and equipment

Laboratory reagents and molecular biology enzymes were obtained from a number of suppliers (Sigma-Aldrich, UK; Invitrogen, UK; VWR International, UK; Bioline, UK; Promega, UK; New England Biolabs, UK or Roche, UK). The source of reagents did not appear to be a critical factor for any experiment unless otherwise specified. Agarose gel electrophoresis equipment was purchased from Thistle Scientific Ltd, UK. DNA markers were obtained from Bioline, UK. Protein gel electrophoresis, semi-dry transfer and gel-drying equipment were obtained from BioRad, UK. Protein markers were obtained from BioRad, UK. All chemicals were dissolved in MiliQ-grade (Milipore, UK) purified water (ddH_2O), adjusted to the appropriate pH using HCl or NaOH and autoclaved at 120°C for 15 minutes or filter-sterilised (0.22μm) unless otherwise stated. Where pH values are indicated these are at 25°C unless otherwise specified. Routine techniques were performed according to standard sources unless otherwise specified (Harlow and Lane, 1988; Sambrook et al., 1989; Ausubel et al., 1999; Freshney, 2000).

2.1.1: Antibodies

Antibody working dilutions were determined empirically for each application and batch of antibody. The following antibodies were used:

c-Myc tag: Clone 9E10; monoclonal mouse IgG_1 antibody raised against amino acids 408-439 of the human $p62^{c\text{-}myc}$ protein fused to KLH (Evan et al., 1985) (Sigma-Aldrich, UK) or clone 4A6, protein G purified mouse monoclonal IgG raised against amino acids 410-420 of human $p62^{c\text{-}Myc}$ (Upstate, UK).
FLAG tag: Clone M2; affinity purified mouse monoclonal IgG_1.
GFP: Living colours anti-Green Fluorescent Protein. Affinity purified mixture of rabbit polyclonal IgG raised against *Aequorea victoria* GFP peptides (Clontech, UK).
GST: Polyclonal goat (Amersham-Pharmacia, UK).
HA tag: Clone HA-7; monoclonal mouse IgG_1 antibody raised against amino acids 98-106 of human influenza virus haemagglutinin fused to KLH (Sigma-Aldrich, UK) or clone 3F10; affinity-purified monoclonal rat antibody raised against amino acids 76-111 of X47 haemagglutinin 1 fused to KLH (Roche, UK).
IG2: Anti-chick PTPσ, raised against a GST-fusion containing the first 2 PTPσ Ig domains (Stoker et al., 1995a).

Phosphotyrosine: Clone 4g10; His-tag purified monoclonal mouse $IgG_{2bκ}$ antibody raised against phospho-tyramine-KLH (Upstate, UK).

2.1.2: Common solutions

Tris-buffered saline (TBS) – 150mM NaCl, 25mM Tris.HCl pH 7.50

TBST – TBS + 0.2% Tween-20

Phosphate buffered saline (PBS) – 130mM NaCl, 15mM Na_2HPO_4, 15mm NaH_2PO_4 pH 7.50 (Not for use in tissue culture)

25xSSC – 0.75M NaCl, 0.375M Na Citrate dihydrate, pH 7.00

2.2: Molecular Biology

2.2.1: Preparation and analysis of DNA

2.2.1.1: DNA purification

Where appropriate, DNA was purified by extraction with phenol-choloroform as decribed previously by equilibration of DNA (<1μg/μl) in Tris-EDTA buffer (10mM Tris.HCl; 1mM ethylenediaminetetraacetic acid (EDTA), pH 8.00 – TE) with buffered phenol/chloroform/isoamylalcohol ($25:24:1_{(v/v/v)}$; pH 8.00) (Sambrook et al., 1989; Ausubel et al., 1999).

2.2.1.2: DNA precipitation

DNA precipitation from aqueous solutions was performed as described previously using 3M sodium acetate pH 5.20 (Routine applications) or 4M ammonium acetate pH 4.80 (Removal of unincorporated nucleosides/oligonucleotides) (Sambrook et al., 1989; Ausubel et al., 1999).

2.2.1.3: Precipitation using an inert carrier

When the DNA concentration was low (<1μg/ml) or contained small DNA molecules (<500bp), precipitation efficiency was increased using the inert carrier, linear polyacrylamide (LPA) (Gaillard and Strauss, 1990). Briefly, 5% acrylamide (without bisacrylamide) was dissolved in 40mM Tris.HCl pH 7.80, 20mM sodium acetate, 1mM EDTA. Linear polymerisation was initiated with 0.1% ammonium persulphate and 0.1%

N,N,N',N'-Tetramethylethylenediamine (TEMED). After 30 minutes, linear polyacrylamide was precipitated with 2.5 volumes of 100% ethanol and centrifugation for 5 minutes at 13k rpm. The resulting pellet was redissolved in 20 volumes ddH_2O on a shaking platform overnight. The 2.5μg/μl LPA solution is stable at 4°C and 4-8μl LPA (10-20μg) was used per precipitation.

2.2.1.4: DNA purification with silica

DNA was extracted from agarose gel slices and from solution using a silica suspension (glass milk) as previously described (Vogelstein and Gillespie, 1979; Boyle and Lew, 1995). Silica (5g) was suspended in 50ml phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4 – PBS). The silica was allowed to settle for 2 hours and the supernatant was then discarded. This step was repeated twice and the final silica pellet was resuspended in 50ml 3M sodium iodide (100mg/ml) and stored at 4°C. The binding capacity of the final solution is approximately 0.5μg/μl. The agarose gel slice or solution containing DNA was incubated at 55°C for 5 minutes in three volumes of 6M sodium iodide with 10μl of silica suspension. The sample was then incubated on ice for 10 minutes. The silica was then pelleted and the supernatant discarded. The pellet was resuspended three times in wash buffer (10 mM Tris.HCl, pH 7.05, 50 mM NaCl, 2.5 mM EDTA, 50% ethanol) and recovered by centrifugation. A final wash was carried out in 70% ethanol. The pellet was air-dried and incubated in 10μl prewarmed TE at 55°C for 10 minutes. The silica was then pelleted and the supernatant containing the DNA carefully transferred to a clean tube.

2.2.1.5: Preparation of oligonucleotide linkers

Complementary oligonucleotide linkers (2nmol each) were annealed immediately prior to use by heating to 90°C for 5 minutes in New England Biolabs Buffer 1 (1x – 10mM Bis-Tris Propane.HCl pH 7.00, 20mM $MgCl_2$, 1mM dithiothreitol) containing 10mM $MgCl_2$ in a hot block, which was then allowed to cool gradually to room temperature.

2.2.1.6: DNA electrophoresis

DNA was analysed by electrophoresis using horizontal agarose gels in Tris-Acetate electrophoresis buffer (40mM Tris acetate, 2mM EDTA). DNA samples were mixed with 10x loading buffer (20% Ficoll 400, 0.1M EDTA pH 8.00, 1% SDS, 0.25% bromophenol blue/xylene cyanole/orange G) to permit sample loading.

2.2.2: Enzymatic manipulation of DNA

2.2.2.1: Restriction digestion, Filling-in, Dephosphorylation

DNA for modification was diluted in ddH_2O or precipitated and resuspended in 10mM Tris.HCl pH 8.00 as required. All digestions were carried out in the presence of 0.1mg/ml bovine serum albumin (BSA). All reactions were performed according to the manufacturer's instructions using established techniques (Sambrook et al., 1989; Ausubel et al., 1999).

2.2.2.2: Ligation

Ligations were performed using 10ng vector, an appropriate amount of insert (See below) and 1-3U T4 DNA ligase in ligation buffer (1x: 66mM Tris pH 7.60, 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 7.5% Polyethylene glycol (PEG) 6000). Molecules with cohesive ends were incubated together overnight at a molar ratio of 3:1 at 18°C. Molecules with blunt ends or single adenine overhangs (i.e. PCR products from non-proofreading polymerases) were incubated overnight at a 1:1 molar ratio at 4°C. The relative amounts of insert and vector were chosen to keep the total DNA concentration below 5ng/µl. Routinely 1µl of the ligation reaction was transformed into competent bacteria.

2.2.2.3: Tailing PCR products for insertion into T-vectors

PCR products produced with a *Taq*-like polymerase produce often have single, template-independent 3' deoxyadenosine base overhangs (Clark, 1988). However, unless freshly generated, purified PCR products were retailed with deoxyadenosine prior to AT cloning using *5U Taq* polymerase for 2 hours at 75°C in 1xTaq buffer (Manufacturer supplied) containing 2mM Mg_2SO_4 and 1mM deoxyadenosine triphosphate (dATP) (Ausubel et al., 1999). The tailed PCR product was then gel purified and cloned into the commercial pGEM T vector series or the pT-Not vector (Gift from N. Dear) (Franz and Dear, 1998).

2.2.3: Polymerase chain reaction techniques

Oligonucleotide primers were ordered from Thermoelectron GmBH, Ulm, Germany. Polymerase chain reactions were carried out using thermal cyclers from MJ Research Ltd, UK. Primer sequences are given in the text with relevant restriction sites underlined for clarity.

2.2.3.1: Polymerase chain reaction (PCR)

PCR (Saiki et al., 1985) using either conventional or degenerate oligonucleotide primers were carried out as follows. Reactions (50µl total volume) were assembled on ice and comprised 100pmol each primer, 1pg vector template, 200µM each dNTP, 1x*Taq* buffer (Manufacturer supplied), 1.5mM $MgCl_2$ (Most reactions) and 0.75U *Taq* polymerase. Where fidelity was a concern, 2.5U *Pfu* polymerase was used with the appropriate buffer and 2mM $MgSO_4$ instead. All other reaction constituents were unchanged. 1M (final) Betaine was frequently added to PCR reactions to improve the efficiency of difficult amplifications. Cycle parameters were optimised for each reaction but were broadly as follows: initial denaturation 94°C 1 minute; 25-35 cycles of 96°C 10 seconds, ((Primer Tm)-5°C) 15 seconds, 72°C 1 minute/kb expected product; reactions finished with a final extension of 10 minutes. When using *Pfu* polymerase, extension was at 68°C not 72°C and the extension time was 2min/kb product with a final extension twice the length of the main extension step (at least 20min). Proteinase K digestion was used occasionally to improve the cloning efficiency of PCR products (Crowe et al., 1991). Completed PCR reactions (50µl) were diluted to 200µl with ddH_2O, SDS ($0.5\%_{(w/v)}$ final) and EDTA (5mM final). Digestion were performed using 20µg Proteinase K at 55°C for 30 minutes. The DNA was then recovered by phenol-chloroform extraction and ethanol precipitation.

2.2.3.2: Site-directed mutagenesis

A site-directed mutagenesis method was developed that was essentially similar to one subsequently published (West and Wilson, 2002). Standard *Pfu*-based PCR with primers 250-350bp apart, one of which contained the desired mutation, was used to generate a PCR product containing the desired mutation. Following purification with agarose gel electrophoresis and silica extraction, half of the purified PCR product acted as a megaprimer in a second *Pfu*-based PCR reaction. Agarose gel electrophoretic analysis of $1/10^{th}$ of the second PCR product confirmed whole plasmid amplification and 10U *Dpn*I added directly to the remaining product selectively digested methylated template DNA leaving only mutated product (37°C 2 hours). Transformation of an appropriate amount of the digested PCR product into competent *Escherichia coli* (*E.coli*), recovered colonies containing the mutant DNA. Restriction analysis (where appropriate) and dideoxynucleotide chain terminator DNA sequencing (2.2.3.4) of coding sequences confirmed the presence of the desired mutation only.

2.2.3.3: Long-accurate polymerase chain reaction (LAPCR)

The chicken LAR cDNA was amplified from RNA using the following long-accurate-PCR (LAPCR) conditions (16:1 *Taq*: *Pfu* ratio; W. Barnes, personal communication). Each reaction was carried out in a final volume of 50µl containing the following components: 100pmol each primer; 0.2mM dNTPs; 1x*Taq* buffer (16mM $(NH_4)_2SO_4$; 67mM Tris-HCl, pH 8.80; 0.01% Tween-20);1.5mM $MgCl_2$; 1.3M Betaine; 1.3% Dimethyl sulphoxide; 1µl RT reaction; 5U *Taq*; 0.3125U *Pfu*. PCR was carried out for 35 cycles using the following profile: 1 minute denaturation at 94°C; 35 cycles of 94°C for 10 seconds, 62.7°C for 10 seconds, 68°C for 7 minutes; final extension at 68°C for 15 minutes. Reactions were not overlaid with mineral oil, thin-walled PCR tubes were used and the machine lid was heated to 96°C.

2.2.3.4: DNA sequencing

DNA was sequenced using the dideoxynucleotide method (Sanger et al., 1977) with the DYEnamic ET dye terminator system (Amersham, UK) and a Megabace 500 automated DNA sequencer according to the manufacturer's instructions. Plasmid DNA and primers for sequencing were prepared in ddH_2O. Reactions (2µl Megabace sequencing mix, 6µl Better Buffer (Microzone, UK), 3.6pmol oligonucleotide primer, 150fmol DNA template) were cycled 30 times (95°C 20 seconds; 50°C 15 seconds; 60°C 1 minute) before precipitation with 2µl 7.5M ammonium acetate and 55µl absolute ethanol followed by centrifugation at 13k rpm for 15 minutes at room temperature. The resulting pellet was washed with 200µl 70% ethanol and air-dried. Samples were resolublised in 10µl loading buffer (Manufacturer supplied) immediately before use. Sequence runs were performed using the long sequence preprogrammed protocol.

2.2.4: Escherichia coli methods

Several bacterial strains were used for the work contained in this thesis. Most work was carried out using the *E.coli* strains: DH5α-FT (Invitrogen, UK), XL1 or XL10-Blue (Stratagene, UK). GST protein expression was carried out in the *E.*coli strain BL21. Standard molecular biology techniques were used unless otherwise specified (Sambrook et al., 1989; Ausubel et al., 1999).

Genotypes: **DH5α-FT:** F′ 80dlacZ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 - thi-1 gyrA96 relA1/F′ proAB+ lacIqZM15 Tn10 (tetr); **XL1-Blue:** *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lac*IqZΔ*M15* Tn*10* (Tetr)]; **XL10-Gold:** TetrD(*mcrA*)*183* D(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lac*IqZD*M15* Tn*10* (Tetr) Amy Camr]; **BL21:** F-, *omp*T, *hsd*S_{β}(r_{β}-m_{β}-), *dcm*, *gal*, (DE3) tonA

2.2.4.1: E.coli propagation: Media and antibiotics

Lysogeny broth (LB) – Miller's formulation (Bertani, 1951; Miller, 1972): For one litre: 10g tryptone, 10g NaCl, 5g yeast extract. (Agar plates +15g agar/l).

LB top agar: For 100ml: 1g tryptone, 1g NaCl, 0.5g yeast extract, 0.7g agar.

Super-optimal Broth (SOB)/Super-optimal broth catabolite repression (SOC) (Hanahan, 1983): For one litre SOB: 20g tryptone, 5g yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$. Add Mg^{2+} salts before use, after autoclaving. SOC : Prepare SOB. Add 20mM glucose with Mg^{2+} salts.

Antibiotics (Working concentrations): Ampicillin (50-100μg/ml); Tetracycline (12μg/ml); Kanamycin (30μg/ml); Chloramphenicol (20μg/ml)

2.2.4.2: Preparation of competent E.coli

Competent *E.*coli were prepared as previously described (Inoue et al., 1990). Untransformed *E.coli* were plated overnight at 37°C and ten to twelve colonies were used to innoculate 250 mL SOB medium in a 2-liter flask. The culture was grown to an A_{600} of 0.6 at room temperature with vigorous shaking (200-250 rpm). The culture was then transferred to a prechilled 500 mL centrifuge bottle on ice and allowed to rest for 10 minutes. The bacteria were recovered by centrifugation at 2500 x g for ten minutes at 4°C. and resuspended in 80 mL ice-cold transformation buffer (TB: 10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$ and 250 mM KCl; pH adjusted to 6.70 with KOH before addition of $MnCl_2$; TB was sterilised by filtration). The bacteria were then allowed to rest on ice for ten minutes and spun down as above. The pellet was resuspended in 20 mL ice-cold TB and dimethylsulphoxide (DMSO) was added ($7\%_{v/v}$ final). The suspension was then incubated

on ice for ten minutes, aliquoted into single use 200µl aliquots and snap frozen in liquid nitrogen for long-term storage at -80°C.

2.2.4.3: Transformation of competent E.coli with plasmid DNA

Bacterial transformation was performed as previously described (Inoue et al., 1990). Competent cells were thawed at room temperature and transferred into a prechilled 15ml polypropylene tube (Falcon 2059) on ice. 1-5 µl of plasmid were added to the competent cells and they were incubated on ice for 30 minutes. They were then heat-pulsed without agitation at 42°C for 30 seconds and replaced on ice for a further 5 minutes. After 0.8ml of SOC was added, the tubes were securely capped and placed horizontally in an incubator at 37°C and shaken vigorously for one hour. An appropriate amount of the mixture was plated onto selective LB plates. Plates were incubated overnight at 37°C.

Blue white screening was used where the cloning strategy permitted. Before plating transformed bacteria, 50µl 20mg/ml 5-bromo-4-chloro-3-indoyl-galactoside (X-gal) in *N,N* dimethyl formamide was spread onto each 10cm^2 agar plate and allowed to air dry. Isopropyl-1-thio-β-D-galactoside (IPTG) was used to induce β-galactosidase expression in E.coli strains expressing the omega fragment of β-galactosidase (XL-1/10 (*lacZ*$^+$)). It was not required in strains that did not express the omega fragment (DH5α-FT (*lacZM15)).* After application of X-Gal, 30µl 100mM IPTG was spread onto each 10cm^2 agar plate and allowed to air dry. Bacteria were then plated in the normal way.

2.2.4.4: Preparation of frozen E.coli stocks

1ml from an overnight culture was supplemented with 1ml 7%$_{(v/v)}$ DMSO. The culture was transferred to a cryotube and frozen rapidly by immersion in liquid nitrogen. Frozen stocks were stored at -80°C for an indefinite period together with an accompanying DNA pellet.

2.2.4.5: Extraction of DNA from transformed E.Coli

Small, low quality and larger-scale, high quality methods of DNA preparation were routinely used to produce DNA for rapid analysis or transfection and sequencing respectively. Commercial alkaline lysis DNA preparation kits (Qiagen, UK; Amersham, UK) were used when problems were encountered with these techniques. All of these protocols use modifications of the alkaline lysis method (Birnboim and Doly, 1979). In

addition, in the high quality DNA preparation, polethylene glycol (PEG) is used to precipitate DNA (Lis and Schleif, 1975).

Minipreps were prepared from 1.5ml overnight cultures of transformed *E.coli* (Zhou et al., 1990; Xiang et al., 1994). The supernatant was removed after 1 minute centrifugation at 13k rpm and the pellet resuspended in the remaining 30-50µl of media. 300µl TENS solution (TE with 0.1M NaOH, 0.5% SDS; made fresh) was added and the sample was mixed gently by inversion before incubation at room temperature for 5 minutes. TENS solution was neutralised by addition of 150µl 3M sodium acetate pH 5.20. The resulting suspension was mixed gently by inversion and cleared by centrifugation at 13k rpm for 5 minutes. The supernatant containing plasmid DNA was transferred to a fresh tube and precipitated by addition of 900µl ice-cold ethanol followed by gentle mixing and centrifugation for 5 minutes at 13k rpm. The pellet was washed in 70% ethanol and the pellet was recovered after a final centrifugation for 2 minutes at 13k rpm. The pellet was air-dried and resuspended in 40µl TE warmed to 70°C. 4µl DNA solution was used per restriction digest or 1µl of a 1:100 dilution was used as template in PCR reactions. RNA present in the final product was removed with 5µl RNAse A solution (0.5mg/ml) added for the final 15 minutes of each restriction digest.

High quality DNA preps were performed as follows. Overnight *E.coli* cultures were pelleted by centrifugation at 3600 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended in GTE buffer (0.2ml/10ml culture; 50mM glucose, 25mM Tris.HCl pH 8.00, 10mM EDTA). Cells were lysed by addition of lysis buffer (0.3ml/10ml culture; 0.2M NaOH, 1% SDS). The resulting solution was mixed rapidly by inversion until the solution cleared and incubated on ice for 5 minutes. Lysis buffer was neutralised by addition of 3M potassium acetate pH 4.80 (0.3ml/10ml culture). The resulting suspension was mixed rapidly by inversion until the solution became less viscous and a fine white precipitate formed. The solution was then incubated on ice for 5 minutes. Cellular debris was removed by centrifugation at 3600 rpm for 15 minutes. The supernatant was transferred to a clean tube and residual RNA was removed by treatment with 0.5mg/ml RNAse A solution (32µl/10ml culture) at 37°C for 20 minutes. The sample was then extracted twice with chloroform (400µl/10ml culture). The DNA was precipitated by addition of an equal volume of isopropanol and immediate centrifugation at 13k rpm at room temperature. The resulting DNA pellet was washed in 70% ethanol and resuspended in ddH_2O (32µl/10ml culture). DNA was precipitated a second time by addition of 4M NaCl (8µl/10ml culture) and 13% PEG8000 (40µl/10ml culture). The sample was

incubated on ice for 20 minutes and then precipitated DNA was pelleted by centrifugation at 13k rpm for 15 minutes at 4°C. The supernatant was decanted and the pellet was washed twice with 70% ethanol. The pellet was recovered following a final centrifugation of 5 minutes at 13k rpm at room temperature. The pellet was air-dried and resuspended in an appropriate amount of TE or ddH_2O.

2.2.4.6: Screening colonies for plasmid size differences indicating insert presence

Colonies from ligations were rapidly screened for inserts using a modified method (Law and Crickmore, 1997; Beuken et al., 1998). 150µl from an overnight culture was pelleted by centrifugation for 20 seconds. The pellet was resuspended in 40µl prewarmed lysis buffer (10% sucrose, 0.25% SDS, 100mM NaOH, 60mM KCl, 0.025% bromophenol blue) and incubated at 37°C for 5 minutes. The solution was placed on ice for 5 minutes and debris was pelleted by centrifugation at 13k rpm for 1 minute. 20µl was loaded onto an agarose gel. Plasmid DNA obtained in this way can be further analysed by restriction digests if the remaining sample is first phenol-chloroform extracted and precipitated.

2.2.4.7: Library screening

Membrane replicas were made from library plates of *E.Coli* transformed with a plasmid library containing embryonic chick cDNAs (R. Aricescu). The replicas were then prepared for screening. Filters (Hybond N+, Amersham-Pharmacia, UK) were incubated serially for two minutes each in Solution 1 (0.2M NaOH, 1% SDS, Solution 2 (0.5M NaOH, 1.5M NaCl) and solution 3 (1.5M NaCl. 0.5M Tris pH 7.50). Bacterial debris was then rubbed off the plates in Wash solution (2xSSC, 0.1% SDS) and filters were rinsed in 5xSSC (10 minutes). Filters were then air-dried on paper towels and the DNA was crosslinked to the filter using the automatic setting on a Stratalinker UV crosslinker (Stratagene, UK).

Membranes were soaked in 0.25M Na_2PO_4 pH 7.20 for followed by pre-hybridisation for 1 hour at 65°C in Hybridisation buffer (O.25M Na2PO4 pH 7.20, 7% SDS, 1mM EDTA, 100µg/ml Poly-A RNA). A chicken LAR EST was identified in a chick bursal library (http://pheasant.gsf.de/DEPARTMENT/dt40.html) by comparison to species homologues. A plasmid containing this cDNA (Clone: dkfz426_27i10r1) was generously provided by J.M. Buerstedde. The insert was excised and labelled using the Brightstar Psoralen-Biotin Nonisotopic labelling kit (Ambion, UK) according to the manufacturer's instructions.

Labelled probe was heat denatured by ten-fold dilution in 10mM EDTA pH 8.00 and incubation at 90°C for ten minutes. Denatured probe (10ng/ml final) was added to fresh pre-warmed (65°C) Hybridisation buffer that was then added to the filters, which were then incubated overnight at 65°C. Following overnight hybridisation, filters were rinsed in 2xSSC, 1%SDS at room temperature (2x5 minutes) and then washed in 0.1xSSC, 1%SDS at 65°C (2x15 minutes). Finally, filters were washed at room temperature in 1xSSC (2x5 minutes).

Bound probe was then detected as follows. Filters were washed in blocking solution (PBS+0.2% Casein, 0.5% SDS; 2x5 minutes), followed by incubation in blocking solution (1x15min). The filters were then incubated with streptavidin-alkaline phosphatase diluted 1:10,000 in blocking solution for 1 hour. Unbound antibody was removed by washes in blocking solution (1x5min) then wash solution (PBS+0.5% SDS; 3x5min). This was followed by incubation in assay buffer (2x2min). Finally, the filters were detected by a five minute incubation in assay buffer (20mM Tris pH 9.80, 1mM $MgCl_2$) containing a 1:50 dilution of CDP-Star (Stock solution: 12.5mM; Applied Biosystems, UK). Detection of chemiluminescent signal from detected library replica filters was performed in the same way as for Western blotting (2.5.4.2). Exposures lasting 20 and 100 minutes were made.

2.2.4.8: Induction of GST fusion protein expression

Bacteria transformed with pGEX vectors encoding inducible GST fusion proteins were induced to express PTPσ proteins by the addition of IPTG (final concentration: 0.1mM) to cultures (OD_{600}=0.6). Induced cultures were harvested after incubation for 4 h at 25°C and lysed by boiling in an appropriate volume of 1xLaemmli buffer containing 5% β-Mercaptoethanol.

2.2.5: RNA methods

Where appropriate, solutions used for RNA analysis were treated with diethylpyrocarbonate (DEPC – 0.2%) (Ausubel et al., 1999).

2.2.5.1: RNA isolation

RNA was extracted from whole chick embryos (Embryonic day 7) using Trizol reagent (Invitrogen, UK) according to the manufacturer's instructions. This is a modification of the original guanidium thiocyanate technique (Chomczynski and Sacchi,

1987). The resulting RNA pellets were solubilised in 100% formamide and stored at -80°C. RNA integrity was confirmed using chicken glyceraldehyde-phosphate-dehydrogenase primers (F – gatggtgaaagtcggagtca; R – gacacccatcacaaacatgg; P. O'Neill, personal communication).

2.2.5.2: Reverse transcription

Reverse transcription (RT) reactions were performed using ImProm-II (Promega, UK) or Transcriptor (Roche, UK) reverse transcriptase (RT). The following conditions were used to amplify the LAR cDNAs. 1µl RNA (100mg/ml), 1µl oligo dT^{17} adaptor (gactcgagtcgacatcgattttttttttttttttvn), 2µl 10mM dNTP mix, 1x RT buffer (supplied with enzyme). Reactions were heated to 65°C for 5 minutes to denature the RNA and then placed on ice for 5 minutes. 0.5µl RNAse inhibitor (10U) and 0.5µl RT (5U) were added and the reactions were incubated at 55°C for 30 minutes.

2.3: Vectors

A number of DNA constructs were made during the work described in this thesis. These are described here and vector maps are appended (Appendix 2: Plasmid vector maps). Where vectors were not generated by the author of this thesis, this is clearly indicated. The numbering of PTPσ residues is based on the Genbank CRYPα1 cDNA sequence, accession number L32780 and its 1,499 amino acid open reading frame (Stoker, 1994). For historical reasons, catalytic site mutations are referred to both by analogy to the equivalent sites in the single PTP1B catalytic domain and by their position within PTPσ; all other mutations are identified by virtue of their PTPσ location.

2.3.1: pCRYPa1

All of the PTPσ constructs generated during this thesis were derived from the chick PTPσ coding sequence in the vector pCRYPα1. This vector was generated by Dr Andrew Stoker and has previously been described. It comprises chick PTPσ1 cloned into the commercial cloning vector pT7/T3α18 (Invitrogen, UK) (Stoker, 1994). This vector also contains a large (ca. 2000 bp), so-far incompletely sequenced 3' untranslated region from chick PTPσ.

2.3.2: pBGA1

Eukaryotic cells transfected with this vector were used to generate PTPσ RAP probe without the 3xFLAG tag for comparison with the protein generated by the p3xFLAG-CMV25-PTPσ-EC-AP construct (Below). The construction of this vector has previously been described (Haj et al., 1999; Chilton, 2000) and encodes wild type PTPσ including the endogenous signal peptide and the complete extracellular domain (bp: 112-2274; aa: 1-721).

2.3.3: p3xFLAG-CMV25-PTPσ-EC

The PTPσ extracellular domain, lacking the endogenous signal peptide (bp: 196-2682 inclusive; aa: 29-829), was amplified by PCR with primers that inserted unique 5'-*NotI* and 3'-*XbaI* sites (F: atatgcggccgcggaaagtccccctgtgttc; R: attctagagggcccaatgacccagat). The PCR product was digested with *NotI* and *XbaI* and inserted into *NotI/XbaI* digested p3xFLAG-CMV25-Myc (Sigma) to produce p3xFLAG-CMV25-PTPσ-EC. Transfection of mammalian cells with this construct results in the secretion of an amino-terminal 3xFLAG tagged PTPσ extracellular domain into the tissue culture medium. Membrane expression of proteins carried by the p3xFLAG-CMV25 backbone is driven by the preprotrypsin signal peptide.

2.3.4: p3xFLAG-CMV25-PTPσ-EC-AP

In order to evaluate the effect of the 3xFLAG tag on PTPσ ligand binding, the p3xFLAG-CMV25-PTPσ-EC-AP vector was constructed by cloning the *BglII-XbaI* fragment from pBGA1 into similarly digested p3xFLAG-CMV25-PTPσ-EC. Eukaryotic cells transfected with this vector were used to generate PTPσ RAP probe with the 3xFLAG tag for comparison with the protein generated by the pBGA1 construct. The p3xFLAG-CMV25-PTPσ-EC-AP vector encodes the complete extracellular domain of wild type PTPσ minus the endogenous signal peptide (bp: 195-2275; aa: 29-721) with an amino-terminal 3xFLAG tag and a carboxyl-terminal alkaline phosphatase moiety.

2.3.5: p3xFLAG-CMV25-PTPσ-UTR

A *BglII/XbaI* fragment (bp 1265 – 4613, plus approximately 1300bp of unsequenced, endogenous PTPσ 3'-untranslated region (UTR)) was obtained from the pCRYPα1 vector (Stoker, 1994). This fragment was inserted into *BglII/XbaI* digested p3xFLAG-CMV25-PTPσ-EC to produce p3xFLAG-CMV25-PTPσ-UTR. Transfection of mammalian cells

with this construct results in the expression of full-length PTPσ (aa: 29-1499) with an amino-terminal 3xFLAG tag.

2.3.6: p3xFLAG-CMV25-PTPσ-Myc

The carboxy-terminal region of PTPσ (bp: 3651-4608; aa: 1232-1499) was amplified by PCR (F – gaactacatggtacagacagaagaccagtacagc; R - cctctagatgttgcatagtgatcaaaacttccc). The reverse primer introduced a unique *XbaI* site. The PCR product was digested with *EcoRI* and *XbaI* and inserted into *EcoRI/XbaI*-digested pCMV25-PTPσ-UTR to create p3xFlag-CMV25-PTPσ-Myc. Transfection of this construct into mammalian cells results in the expression of full-length PTPσ (aa 29-1499) with an amino-terminal 3xFLAG tag and a carboxy-terminal Myc tag.

2.3.7: p3xFLAG-CMV25-Myc-5'+3'XhoI

The 3xFLAG-PTPσ-Myc cassette was made excisable by insertion of two *XhoI*-containing linkers, one each into the p3xFLAG-CMV25-Myc *SacI* (5') and *BamHI* (3') sites. The *NotI-XbaI* fragment from p3xFLAG-CMV25-PTPs-Myc was then inserted to create the final vector (5' *XhoI* F – tcgctcgagtcttagct; 5' *XhoI* R – aagactcgagcgaagct; 3' *XhoI* F – gatcgtactcgagcta; 3' *XhoI* R – gatctagctcgagtac). The intermediate constructs p3xFLAG-CMV25-PTPσ-Myc-5'*XhoI* and p3xFLAG-CMV25-PTPσ-Myc-3'*XhoI* were kept and used in other cloning steps.

2.3.8: p3xFLAG-CMV25-PTPσ-3xMyc

p3xFLAG-CMV25-PTPσ-3xMyc was derived from p3xFLAG-CMV25-PTPσ-Myc by insertion of a linker containing an additional two Myc tags into *XbaI*-digested vector. Insertion of this linker destroyed one of the two resulting XbaI sites to recreate a single *XbaI* site, located between the PTPσ sequence and the tag sequence (F – ctagagaacaaaaactaataagtgaagaagacctagagcagaagctgatcagcgaggaggacctga; R – ctagtcaggtcctcctcgctgatcagcttctgctctaggtcttcttcacttattagtttttgttct).

2.3.9: p3xFLAG-CMV25-PTPσ-HA

This vector was derived from the p3xFLAG-CMV-Myc-3'*XhoI* vector by linker insertion (HA F – ggatctagataccctacgacgtgcccgactacgcctgactcgagagc; HA R – gctctcgagtcaggcgtagtcgggcacgtcgtaggggtatctagatcc) and replacement of the single Myc tag

with an HA tag. The p3xFLAG-CMV25-Myc-3'*XhoI* vector and the annealed linkers were digested with *XbaI* and *XhoI*. Linker insertion replaced the Myc tag with an HA tag in frame with the PTPσ insert and recreated both the 5' *XbaI* site and the 3' *XhoI* site. Transfection of mammalian cells with this vector results in the expression of full-length PTPσ with an amino-terminal 3xFLAG tag and a carboxy-terminal HA tag.

2.3.10: pCI-Neo-PTPσ-HA

This vector was created by sub-cloning the *BstEII*/3' *XhoI* PTPσ fragment from p3xFLAG-CMV25-PTPσ-HA-5'+3'*XhoI* and inserting it into the *BstEII*/*SalI*-digested pCI Neo-PTPσ vector (A.Stoker, unpublished). The pCI-Neo-PTPσ vector contains the entire XbaI flanked PTPσ fragment from the pCRYPα1 vector cloned into the pCI-Neo mammalian expression vector (Promega, UK). pCI-Neo-PTPσ-HA therefore contains full-length PTPσ (bp: 112 – 4606 inclusive; aa: 1-1499 inclusive) including the endogenous PTPσ signal peptide but not the preprotrypsin leader used in all of the other mammalian expression vectors in this thesis. Transfection of mammalian cells with this vector results in the expression of full-length PTPσ without an amino-terminal tag, but with a carboxy-terminal HA tag.

2.3.11: pHL vectors – C-terminally His6 tagged protein expression

Eukaryotic expression vectors containing various extracellular domain deletions of PTPσ were created using the pHL vector for dimerisation experiments using dynamic light scattering. The vectors pHL and pHL-FNΔ3 were provided by Dr Radu Aricescu. The construction of the pHL backbone which contains 6 tandem Histidine (His_6) residues has previously been described (Aricescu et al., 2006). pHL-FNΔ2 and pHL-FNΔ4 drive the expression of carboxyl-terminally His_6-tagged truncated PTPσ extracellular domain proteins lacking sequences after the second or fourth FN3 domain. The PTPσ extracellular deletions were based upon those previously characterised by John Chilton (Chilton, 2000). pHL-FNΔ3-AP adds an alkaline phosphatase moiety (lacking the endogenous AP signal peptide – CAP) to the truncated PTPσ extracellular domain product produced by pHL-FNΔ3. Finally, pHL-SAP encodes a soluble form of the alkaline phosphatase domain alone.

The alkaline phosphatase inserts for pHL-SAP and pHL-FNΔ3-AP (CAP) were amplified by PCR (SAP F – cgcggaattcgacccaagcttcctgcatgctgctg; CAP F –

cgcggtaccatcatcccagttgaggaggagaacc; SAP/CAP R – cgcggtacccacccgggtgcgcggcgtcggtg) from pAP-Tag4 (SAP) or pBGα1 (CAP) to introduce either two *KpnI* sites (CAP) or unique 5' *EcoRI* and 3' *KpnI* sites at each end of the product (SAP). The SAP insert was cloned into *EcoRI/KpnI* digested pHL and the CAP insert was cloned into *KpnI*-digested pHL-FNΔ3.

The PTPσ extracellular domain from the signal peptide to the desired deletion sites was amplified (FNΔ2/4 F – cgcggaattcaagcttgcatgcctgcaggtcgact; FNΔ2 R – cgcgggtaccaacctggatggggtcagaaagaggc; FNΔ4 R – cgcgggtaccgacgataactggcgagctctccggg) from the pBAG series of extracellular domain deletions (Chilton, 2000) to create unique *EcoRI* and *KpnI* sites at the 5' and 3' ends of the products, respectively. The resulting appropriately digested inserts were cloned into *EcoRI/KpnI* digested pHL (R. Aricescu).

2.3.12: pGEXKT-PTPσD1+D2

Bacterial expression vectors containing either wild type PTPσ or PTPσ containing substrate trapping mutations (D181A, Q262A, Dbl, C215S, C215D) fused to glutathione-S-transferase (GST) were created using the pGEX-KT vector (Hakes and Dixon, 1992). Induction of *E.coli* transformed with this vector results in the production of a fusion protein where the PTPσ portion is carboxy-terminal to the GST moiety, separated from it by a thrombin site and a 6 residue glycine-rich linker, which facilitates thrombin cleavage. The PTPσ intracellular domain (bp: 2743-4701; aa: 928-1499) was amplified by PCR using primers that created 5' and 3' *BamHI* sites (F – ggtcaggatcccagacagcaaacggaaagatt; R – ggtcaggatcctatgttgcatagtgatcaaaact).

2.3.13: Chicken cDNA libraries

Plasmid libraries containing cDNA derived from embryonic chick RNA were previously created in the laboratory (Aricescu, 2002).

2.3.14: Isolation of chicken LAR cDNA

Two chicken LAR clones were generated independently (pGEM T-easy LAR clone 1 (pLAR-1) and pGEM T-easy LAR clone 2 (pLAR-2)) by ligation of RT-PCR products (LAR 1 F – gtttgatgatgggtcgggat; LAR 1 R – ccggccgctgcgtccgcaaag; LAR 2 F – gtttgatgatgggtcgggatc; LAR 2 R – ctgcgtccgcaaagtcttcac), amplified from an E7 mRNA-enriched template (Oligotex: Qiagen, UK or mini-oligo(dT), 5 Prime Inc, Boulder USA), into the pGEM-T-easy vector (Promega).

2.4: Cell Biology

HEK 293T, and Cos-7 cells were obtained from the American Tissue Culture Collection (ATCC). PC12 cells were a gift of Dr Jonathan Ham, Media and sera were sourced from Sigma-Aldrich, UK. Plasticware was obtained from Techno Plastic Products (TPP) AP, Switzerland. Dulbecco's PBS solution A (DPBSA), which is Ca/Mg^{2+} free, and complete Dulbecco's PBS (DPBS), which contains both divalent cations, were purchased from Sigma. Single batch foetal bovine serum was obtained from Sigma-Aldrich.

2.4.1: Cell propagation

All cell lines were routinely maintained in Dulbecco's modified eagle media (DMEM; High glucose) without antibiotics, supplemented with 10% heat inactivated FBS at 37°C, 5% CO_2. However, for H_2O_2 treatments and related experiments, HEK-293T cells were grown in F10 or F12 media as described in the text and cells were serum starved overnight before treatment. Cells were passaged every 2-3 days using Trypsin/EDTA solution (Sigma-Aldrich, UK) and seeded at a density of $1x10^5$ cells/ml (Gibco recommendations – $15cm^2$ plate: $20ml/2x10^6$ cells; 10cm2 plate: 10ml media/$1x10^6$ cells; 6-well plate: 2ml media/$2x10^5$ cells; 12-well plate: 1ml media/$1x10^6$ cells; 24-well plate: 0.5ml media/$5x10^4$ cells). The progress of trypsinisation was monitored and terminated by the addition of complete growth medium.

2.4.2: Transfection

HEK-293T cells were transfected using two different methods and cells were harvested 48-72 hours post transfection. No significant difference was detected between cells transfected in either way for any parameter including transfection efficiency and PTPσ expression level and cleavage pattern (Data not shown). PC6-3 cells were transfected using Effectene (Qiagen, UK) according to the manufacturer's instructions.

2.4.2.1: Calcium Phosphate transfection

An optimised calcium phosphate transfection method was used (Jordan et al., 1996; Jordan and Wurm, 2004). Cells were plated on the day before transfection as described above. Cells were transfected with a total of 100µl of transfection mix per 1ml of growth medium. DNA (2.5µg/ml medium) was diluted into solution A (250mM $CaCl_2$). An equal volume of solution B (1.4mM Sodium phosphate, 140mM sodium chloride, 50mM Hepes, pH 7.05)

was added to the DNA solution and mixed quickly with the pipette. After 1 minute, the transfection mix was added to the cells and the plates were returned to the incubator. After 4 hours the media was removed and replaced with 10% glycerol in DPBSA. After 1 minute glycerol shock, the glycerol solution was replaced with normal growth medium. The glycerol shock step was omitted for HEK-293T cells.

2.4.2.2: Polyethyleneimine transfection

Polyethyleneimine (PEI; 25kDa branched form, Aldrich 40,872-7) was diluted to 1mg/ml in ddH_2O and used to transfect cells as previously described (Durocher et al., 2002). The resulting solution was adjusted to pH 7.40 with dilute HCl and passed through a 0.22μm filter to sterilise it. The solution was stored as aliquots at -20C. Cells to be transfected were plated at $1x10^5$ cells/ml the day before transfection. Immediately before transfection, the media on the cells was replaced with a half volume of fresh media. The DNA to be transfected was diluted in $1/10^{th}$ media volume serum-free media. For HEK-293T cells in this lab the optimal amount of DNA was 16μg/ml transfection solution and the optimal $DNA_{(\mu g)}$:$PEI_{(\mu g)}$ ratio was 8:9. PEI was added to the DNA and mixed well by vortexing for 10 seconds. The DNA-PEI solution was allowed to rest for 10 minutes at room temperature to allow complex formation. The DNA-PEI mixture was then added to the cells, which were then returned to the incubator. After 3 hours, the media on the cells was made up to the normal volume again.

2.4.3: Polyethyleneimine coating cover-slips

Cells for immunofluorescent analysis were plated onto coverslips before transfection. To optimise adherence, the cover slips were treated with polyethyleneimine (Personal communication, L. Ensor). All stages were carried out under sterile conditions. Polyethyleneimine (Sigma P3143 $50\%_{Aq}$) was diluted to 5% in water and stored at -20°C until required. Prior to use the frozen stock was diluted to 0.05% in 0.15M boric acid. Coverslips were coated overnight in 0.05% PEI, washed twice in ddH_2O and air-dried leaning at an angle on the side of a Petri dish. The Petri dish containing the coated coverslips was then sterilised for 5 minutes in a UV crosslinker (Stratalinker, Stratagene, UK)

2.4.4: Immunofluorescence

Cells for immunofluorescent analysis were cultured on polyethyleneimine-coated coverslips placed into the well of a 6-well plate. After removing the growth media, the coverslips were rinsed three times in cold DPBSA before being fixed for 30 minutes in cold 4% PFA. Following fixation, all steps were carried out at room temperature unless otherwise specified and wash steps were carried out on a gently rocking platform. Fix was removed by a 5 minute wash in PBS followed by quenching in 2% glycine in PBS for 5 minutes. The glycine solution was removed with a further 5 minute wash in PBS. The fixed cells were permeabilised by incubation for 5 minutes in 0.1% TX100, 0.05% SDS in PBS. Excess detergent was removed with 2 washes of 5 minutes each in PBS. The coverslips were then incubated with αHA (1:1000) and α FLAG (1:1000) for 45min at 37°C on parafilm. Coverslips were then returned to the 6-well plates and unbound antibody was removed by 6 washes in PBS. A second incubation step was performed as above using the appropriate α-Rat TRITC conjugated and α Mouse-FITC conjugated antibodies (Both 1:1000; Dako, UK). The cells were washed again as above and the coverslips mounted with Fluorsave (Calbiochem, UK). Immunofluorescence was observed using an Axioskop 2+ microscope with Axiovision software and with exposure times of 1200ms and 2000ms for FITC and Texas Red channels, respectively.

2.4.5: Trypsin treatment of intact monolayers

Trypsin treatment of intact monolayers was performed as previously decribed (Olson et al., 1988). All aspirations were performed gently using a wide bore pipette and an automatic pipettor on the lowest setting. Cells were washed twice with PBS at room temperature before incubation for ten minutes in 5 ml room temperature Trypsin solution (0.5 mg/ml L-1-tosylamido-2-phenylethyl chloromethylketone-trypsin (Sigma, UK) in DPBS; prepared fresh from powder). After ten minutes, the trypsin solution was gently aspirated without disturbing the plate and replaced with 8 ml ice-cold inhibitor solution (1.5 mg/ml soybean trypsin inhibitor (Sigma), 1% (w/v) bovine serum albumin in DPBSA; prepared fresh from powder). This was then gently aspirated without disturbing the plate and the cells were washed with ice-cold DPBS. Cells were lysed in the conventional manner.

2.4.6: Receptor affinity probe (RAP) assay

Receptor affinity probe experiments were performed as previously described using secreted alkaline phosphatase (SEAP) fusion proteins (Haj et al., 1999; Flanagan and Cheng, 2000; Flanagan et al., 2000). Conditioned media from pBGA1/pCMV25-PTPσ-EC-AP transfected HEK-293T cells was removed between 3 days to 2 weeks after transfection, supplemented with 1M Hepes pH 7.00 (Final: 10mM) and sodium azide ($0.1\%_{(w/v)}$ final) and filtered to sterilise the media and remove cells and debris.

The SEAP activity in 100µl conditioned media was determined following heat inactivation of endogenous AP (5 minutes, 65°C) at 37°C using an equal volume of SEAP buffer (21% diethanolamine, 1mM $MgCl_2$). The AP substrate p-Nitrophenylphosphate (pNPP) (10µl) was added and AP activity measured at 405nm (1mU SEAP activity =+0.04 U/minute A_{405}, SEAP specific activity = 2U/µg protein (Aricescu, 2002)).

RAP assays on sections or fixed cells were performed as follows. Fresh-frozen cryosections were washed in wash buffer (0.035% w/v sodium bicarbonate; 20 mM HEPES; 0.05% w/v BSA; pH 7.00) before fixation in 100% methanol at -20°C for 90 seconds. Slides were then briefly washed in wash buffer and excess liquid was removed. The section was covered in AP probe media and incubated at room temperature for 75 minutes. Unbound fusion protein was removed by washing in TBST (5x5min). Bound probe was fixed to the section by a 90 seconds incubation in 4% paraformaldehyde. Slides were again washed and endogenous AP activity was inactivated by incubation at 65°C for 30 minutes. Alkaline phosphatase activity was then detected using NBT/BCIP (Roche, UK) as recommended by the manufacturer. RAP assays on living cells were performed in the same way except that cells were incubated in conditioned media for 8 hours.

2.4.7: Homophilic binding – SDS-PAGE assay

PTPσ homophilic binding was assessed by SDS-PAGE as previously described (Cismasiu et al., 2004). PTPσ- and mock-transfected cells were prepared for the homophilic binding assay . The medium was removed, and prepared as for a RAP probe conditioned medium. It was saved at 4°C for future analysis. The cells were then washed three times in DPBSA and conditioned media was added. The cells were then incubated in conditioned medium for 8 hours at 37°C. The media was again set aside and cells were washed twice in ice cold

DPBSA before lysis in standard lysis buffer. Cell lysates and conditioned media were compared using SDS-PAGE analysis.

2.4.8: Microscopy

Coverslips were analysed following staining and mounting on an Axioskop 2 with Axiovision software (Zeiss) at 400x magnification using phase and immunofluorescence optics. The exposure time was 1.2 seconds for the FITC channel and 2 seconds for the TRITC channel.

2.4.9: Time post transfection assay

HEK-293T cells were plated in $6cm^2$ plates and transfected as described above. All steps were carried out using a single preparation of cells or transfection mix that was used on each plate in turn. Cells were harvested as described below at 2, 4, 8, 16, 32 and 64 hours after removal of the transfection mix and lysates were analysed by SDS-PAGE and western immunoblotting.

2.4.10: Cell density experiment

A single $10cm^2$ dish of HEK-293T cells (~$2x10^5$ cells/ml) was transfected with calcium phosphate as described. Following removal of the calcium phosphate precipitate, cells were allowed to rest for 4 hours. Cells were then gently returned to a single cell suspension by trituration without using trypsin before being replated into the wells of a 6 well plate at the following densities: $1.6x10^4$, $3.12x10^4$, $6.25x10^4$, $1.25x10^5$, $2.5x10^5$, $5x10^5$ cells/ml. Cells were then allowed to express the transfected protein for a further 64 hours before harvesting. Lysates were analysed by SDS-PAGE and western immunoblotting

2.4.11: Treatment of cells with BS_3

Chemical crosslinking was performed by washing cells twice in ice-cold PBS before incubation with 1mM BS_3 (Pierce, UK) in ice-cold DPBS for 60 minutes with gentle agitation. Crosslinking was quenched by washing cells in TBS for 15 minutes. Cells were then lysed nomally.

2.4.12: Treatment of cells with Phorbol-12-myristate-13-acetate (TPA)

Phorbol-12-myristate-13-acetate (TPA) was obtained from Calbiochem Phorbol-12-myristate-13-acetate (TPA) was obtained from Calbiochem, dissolved in DMSO (Final TPA concentration: 1mM) and stored at -20°C until required. TPA was added to cells at the indicated concentration, which were then replaced in the incubator for 30 minutes.

2.4.13: Treatment of HEK-293T cells with pervanadate

Pervanadate was prepared as previously described (Lyons, 2003). 1.84g sodium orthovanadate (Na_2VO_4) was dissolved in 45ml ddH_2O and activated as follows. The pH was adjusted to pH 10.00 with 1M NaOH or 1M HCl as appropriate. The solution was then boiled until colourless and the pH adjusted back to pH 10.00. This was repeated until the pH remained stable and colourless after boiling. The volume was returned to 50ml with ddH_2O. This 200mM stock was defrosted immediately before use at 100°C and vortexed to redissolve any crystals. Working solution was prepared by adding 150µl 200mM activated orthovanadate to 789µl PBS. Hydrogen peroxide (H_2O_2) (30% stock) was diluted 1:10 in PBS (3% final) and stored at 4°C for up to a month. To produce a pervanadate solution (30mM), 61µl 3% H_2O_2 was added to the diluted sodium orthovanadate and mixed well. The mixture was incubated at room temperature in the dark for 15 minutes to allow pervanadate formation. The stock solution was diluted to 1mM in culture media and the resulting 1mM solution was diluted 1:10 into the media on the cells (Final concentration 100µM). Cells were returned to the incubator for 30 minutes before being harvested as described.

2.4.14: Treatment of cells with H_2O_2

HEK-293T cells for H_2O_2 treatment were cultured in Ham's F10 media with 10% FBS. To remove residual serum, cells were washed twice in F10 media without serum. Finally, cells were treated with the indicated amount of H_2O_2 freshly diluted in Ham's F10 media for the indicated period of time. Cells were harvested in the normal manner.

2.4.15: Treatment of cells with serum

HEK-293T cells were grown in Dulbecco's Modified Eagle Media supplemented with 10% FBS. The day before the experiment, conventional growth media was refreshed or replaced

with fresh DMEM lacking any serum and the cells were cultured overnight. On the day of the experiment, the overnight media was replaced with DMEM containing 10% FBS as indicated and the culture dish was replaced in the incubator for the indicated length of time. Cells were harvested as described above.

2.5: Protein analysis

Protein concentrations were determined using Bradford reagent in the micro-assay protocol (Sigma, UK).

2.5.1: Harvesting and lysis of HEK-293T cells to obtain protein samples

Following treatment and harvesting of the tissue culture supernatant if appropriate, HEK-293T cells were washed briefly twice in ice-cold DPBS. Cells were then lysed by the addition of an appropriate volume of lysis buffer (1ml/10cm diameter plate). Samples were left in lysis buffer on ice for 30 minutes with frequent mixing by inversion. Insoluble material was set aside following a 30 minute centrifugation at 13,000 rpm in a refrigerated centrifuge at 4°C. Samples were stored at -20°C until required. Unless otherwise indicated, cells were lysed in the following lysis buffer: 150mM NaCl, 20mM Tris-HCl pH 7.40, 2mM EDTA, 1% Nonidet P-40, 7μg/ml Pepstatin A, 1.5 mg/ml Aprotinin, 2mM PMSF). In indicated experiments a cysteinyl modifying agent (Iodoacetamide 20mM) was added. In experiments where phosphotyrosine levels were analysed, phosphatase inhibitors were added with the protease inhibitors (10mM NaF, 2mM activated sodium orthovanadate).

2.5.2: Deglycosylation of proteins with PNGase F

Peptide N-glycosidase F (PNGase F – Sigma-Aldrich, UK) treatment was carried out as follows. Briefly, protein samples in conventional lysis buffer were denatured at 100°C for 10 minutes in glycoprotein denaturing buffer (0.1% SDS, 1% β-mercaptoethanol, 50mM sodium phosphate pH 7.50). The sample was cooled to room temperature and non-ionic detergent was added (Nonidet-P40, 1% final). The denatured protein was then incubated in a water bath at 37°C for 10 minutes before 500u PNGase F was added. Digestion was allowed to proceed for 3 hours. Samples were analysed by SDS-PAGE as described below.

2.5.3: Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as originally described (Laemmli, 1970). Stacking gels (0.125 M Tris.HCl, pH 6.80; 0.1% SDS) contained 3% total acrylamide ((T); Stock: 30% 37.5:1 total acrylamide:bisacrylamide (BioRad, UK)); the percentage of the resolving gel (0.375 M Tris.HCl, pH 8.80; 0.1% SDS) varied according to the size of the protein of interest (e.g. Disulphide crosslinked full length PTPσ – 6% T resolving gel). Gels were polymerised using TEMED (Final: 0.04-0.1%$_{(v/v)}$) and APS (Final: 1%). Electrophoresis was carried out in electrode buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS; pH~8.30). Samples (10μg total protein) were completely denatured by incubation for 5 minutes at 100°C in sample buffer (2x: 0.125 M Tris.HCl *p*H 6.80, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.001% bromophenol blue). Electrophoresis was carried out with a current of 10mA per gel until the marker left the gel. Protein size was determined by comparison to the migration of Precision Plus Protein prestained standards (BioRad, UK).

2.5.3.1: Two-dimensional Non-reducing/ Reducing SDS-PAGE

Samples (10μg total protein) were analysed as described above except that reducing agents were omitted from the sample buffer and an alternative protein ladder that lacked reducing agents was used (SeeBlue Prestained standard, Invitrogen, UK). A schematic showing the process can be found below (4.3.4). The gel was removed from the electrophoresis cassette and following zinc staining, protein-containing lanes were excised and destained. The resulting sample lanes were incubated in 1x Laemmli buffer (62.5 mM Tris-HCl pH 6.80, 2% SDS, 10% glycerol, 0.001% bromophenol blue, 50mM N-Ethylmaleimide) with or without 5%$_{(v/v)}$ 2-Mercaptoethanol for 5 minutes at 100°C. The excised strips were then washed thoroughly for 2x5 minutes in 1%SDS, 10% glycerol, 62.5mM Tris-HCl pH 6.80, 20mM Iodoacetamide to remove reducing agents and placed in an electrophoresis cassette. A 6%T resolving gel followed by a 3%T stacking gel was poured below or around the gel slice, respectively. An extra spacer was used to form a single well at the side of the gel for protein markers. The gel was then analysed in the usual manner.

2.5.3.2: SDS-PAGE analysis - Neutral silver stain

Silver staining visualisation was performed as previously described (Shevchenko et al., 1996). Briefly, the gel was fixed overnight (50% methanol, 5% acetic acid). The gel was

then washed for 10 minutes with 50% methanol and for 30 minutes in ddH_2O. The gel was sensitised for 1 minute in 0.03% sodium thiosulphate and then rinsed three times for 5 minutes each in ddH_2O. The gel was then incubated in 0.1% silver nitrate for 20 minutes. The gel was then rinsed twice in ddH_20 for 1 minute before development (0.04% formaldehyde, 2% sodium carbonate). Development was stopped by addition of 5% acetic acid. If necessary, gels were destained as required in Farmer's Reducer (12.4g/l sodium thiosulphate, 0.28g/l potassium ferricyanide). Gels were stored in water or dried using a Model 583 gel dryer on the automatic 2 hour step programme (BioRad, UK). To prevent thick or high percentage gel cracking, gels were incubated for 1 hour in 35% ethanol, 2% glycerol prior to drying (Amersham Pharmacia website - Gel Drying Troubleshooting).

2.5.3.3: SDS-PAGE analysis - Brilliant blue stain

Alternatively, SDS-PAGE gels were stained with brilliant blue (Alete, 2003). Gels were incubated in fix (50% Methanol, 10% Acetic acid) for 30 minutes and then incubated in staining solution (50% Methanol, 10% Acetic acid, 0.1% Brilliant Blue G-250; filtered through 3MM paper) for 30 minutes. Gels were washed in water and destained overnight in destain (12% Methanol, 7% Acetic acid) until the required intensity of staining was achieved. Gels were stored in ddH_2O or dried as described above.

2.5.3.4: SDS-PAGE analysis 3- Zinc staining

Proteins were stained using a modification of a published method (Fernandez-Patron et al., 1992). Briefly, gels were removed from the gel cassette and rinsed in ddH_2O for 5 minutes. Then the gels were sensitised in 1% sodium carbonate for 15 minutes. Gels were incubated in imidazole solution (200mM imidazole, 0.1% SDS) for 30 minutes and then rinsed in ddH_2O for 1 minute. Finally, gels were developed in 100mM zinc acetate solution (0.45μm filtered) until they reached the desired opacity. Development was terminated by rinsing the gels in ddH_2O three times for 5 minutes each time. Gels were destained in 50mM EDTA, pH 8.00 prior to protein transfer or 2D electrophoresis.

2.5.4: Western blotting

Proteins separated by SDS-PAGE analysis were transferred to Immobilon-P (Pore size: 0.45μm) polyvinylidene fluoride (PVDF) membranes (Milipore, UK) using a modification of the original protocol (Towbin et al., 1979). Gels were equilibrated in ice-cold transfer buffer (25mM Tris.HCl, 192mM Glycine, 20% methanol (pH~8.30)). 6 pieces of 3MM

paper (Whatman, UK) and one piece of PVDF membrane pre-trimmed to the size of the gel were prepared by incubation in ice-cold transfer buffer. The PVDF membrane was first wetted in methanol. A cassette was assembled on the cathode plate of a Trans-Blot II semidry electrophoretic transfer apparatus (BioRad, UK) according to the manufacturer's instructions. Up to two gels at a time were transferred for 30 minutes at room temperature using 20V constant voltage. After transfer, to enhance protein retention and reduce background noise, membranes bearing the proteins from transferred gels were washed in ddH_2O for 5 minutes and then submerged in methanol and allowed to air dry (Milipore Immobilon-P technical literature). Membranes were rewet in methanol followed by a five minute wash in ddH_2O prior to subsequent analysis.

2.5.4.1: Analysis of proteins on membranes 1- Ponceau stain

Proteins transferred to PVDF were stained for 1 minute using Ponceau S/Ponceau red (10x Stock solution (2% ponceau red, 30% trichloroacetic acid, 30% sulfosalicylic acid); Dilute 1:10 in 1% acetic acid prior to use). The membrane was then rinsed in ddH_2O until the desired contrast was reached. The membrane was destained using frequent changes of ddH_2O. If necessary the final traces of red stain were removed with a wash in 0.1M NaOH.

2.5.4.2: Analysis of proteins on membranes 2 - Immunological detection

Non-specific antibody binding was blocked by incubation of the membrane for 1 hour at room temperature in Tris-buffered saline with Tween (TBST) containing either 10% $_{(w/v)}$ non-proprietary dried, skimmed milk powder (all primary antibodies other than anti-phosphotyrosine) or 3% bovine serum albumin (anti-phosphotyrosine). Membranes were rinsed for 5 minutes in TBST and then detected by incubations of one hour each in the appropriate blocking buffer with primary antibody and subsequently with horseradish peroxidase (HRP)-conjugated species-specific secondary antibody. Following each antibody incubation step, unbound antibody was removed by a series of five 5-minute washes in TBST.

Bound HRP-labelled species-specific secondary was detected using the ECL-Plus system (Amersham) according to the manufacturer's instructions using cassettes with intensifying screens and blue-sensitive autoradiography film (Kodak X-Omat Blue; Kodak, UK). Exposed film was either developed automatically using a Compact X4 film processor (Xograph Imaging Systems, UK) or manually using the X4 developing reagents.

2.5.4.3: Analysis of proteins on membranes 3 - Stripping western blots

Membranes blocked with skimmed milk were stripped to allow a second or third antibody probing in the following manner (Suck and Krupinska, 1996). Blots were rinsed for 5 minutes in ddH_2O and then incubated for 5 minutes in 0.2M NaOH. This was followed by a 5 minute wash in ddH_2O. The membrane was then re-blocked and processed as described above.

2.5.5: Rate-zonal centrifugation

5-20% glycerol gradients were prepared in 4.8ml polyallomer (Beckman, UK) tubes using the underlayering technique and allowing the formed step gradients to diffuse overnight at 4°C. The glycerol solutions were prepared in the gradient buffer (1mM EDTA, 2mM $MgSO_4$, 0.1% Triton X-100, 20mM Hepes-NaOH pH 8.00, 7µg/ml Pepstatin A, 1.5µg/ml Aprotinin, 2µg/ml PMSF). Unfortunately, it was not technically possible to confirm that the gradient formed was linear, although this might be achieved by measuring the refractive index of blank fractions. 100µl protein sample in Triton lysis buffer (1% Triton X-100, 20mM Tris pH 8.00, 150mM NaCl, 7µg/ml Pepstatin A, 1.5µg/ml Aprotinin, 2µg/ml PMSF) was gently applied to the top of the gradient and the samples were centrifuged at 43k rpm in a Beckman Ultracentrifuge using a SW41Ti rotor for 17 hours. Samples (~250µl) were collected from the bottom of the tube using a 27G needle and analysed by SDS-PAGE.

2.5.6: Immunoprecipitation

All steps were carried out on a rotating platform at 4°C unless otherwise specified. Protein A/G (Coupled to agarose; Fast-flow, 2mg/ml as 50% slurry; Upstate, UK) was prepared by addition of BSA (Final: $5\%_{(w/v)}$) to block non-specific binding. Protein A/G was blocked overnight. Protein lysates (adjusted to 2µg/µl) were cleared by incubation for 1 hour with 50µl Protein A/G. The agarose beads were removed by centrifugation (20s, 13k rpm), 20µl precleared lysate was set aside for use as a lysate control. Precleared lysates (400µg) were incubated overnight with 2.5µg specific or control antibodies. The next day, 60µl protein A/G was added and the sample incubated for an additional 2 hours. The immunoprecipitate was collected by centrifugation for 30s at 13k rpm and washed five times by vigorous resuspension in lysis buffer followed by centrifugation as above. Finally,

immunoprecipitates were eluted from the beads by denaturation in 50µl 2xLaemmli buffer at 100°C for 5 minutes followed by centrifugation as above. The supernatant was removed and 15µl was analysed by SDS-PAGE.

2.6: Bioinformatics

DNA and protein analysis was performed using the programs BioEdit, Chromas, and pDraw32, which the authors have generously made freely available to download (http://www.mbio.ncsu.edu/BioEdit/bioedit.html; http://www.acaclone.com/; http://www.technelysium.com.au/chromas.html) (Hall, 1999; Tippmann, 2004).

2.6.1: Transmembrane domain prediction

Transmembrane peptides were predicted using the THMM server as described (Sonnhammer et al., 1998; Krogh et al., 2001; Moller et al., 2001).

2.6.2: Molecular weight prediction

The tool, Compute pI/Mw, from the Expasy site (http://ca.expasy.org/tools/) was used to predict the molecular weight of the peptide backbone of all PTPσ species (Gasteiger E et al., 2005). Molecular weights were computed using average amino acid molecular weights rather than monoisotopic weights.

2.6.3: Protein O-/N-glycosylation prediction

Protein N- and O- glycosylation was predicted using the NetOGlyc and NetNGlyc tools (http://www.cbs.dtu.dk/services/NetNGlyc/ and ~/NetOGlyc/ (Gupta et al., 2002; Julenius et al., 2005).

2.6.4: Tyrosine sulphation prediction

Tyrosine sulphation prediction was performed using Sulphinator (http://www.expasy.org/tools/sulfinator/) (Monigatti et al., 2002).

2.6.5: Hydrophobicity analysis

Hydrophobicity analysis was performed using the method of Kyte & Doolittle (Kyte and Doolittle, 1982) in the BioEdit package.

2.6.6: Helical wheel plots

Helical wheel plots were generated using the Java applet at http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html.

2.6.7: Chicken EST database searches

During the course of the work described in this thesis three chicken EST databases were generated that were useful in the cloning of chicken LAR http://www.chick.umist.ac.uk/; http://www.chickest.udel.edu/; http://pheasant.gsf.de/DEPARTMENT/dt40.html (Boardman et al., 2002; Carre et al., 2006).

Chapter 3: Overexpression studies of PTPσ

The core chapters of this thesis examine the role that dimerisation plays in PTPσ function (Chapter 4, Chapter 5). In order to carry out these experiments it was necessary to create a number of constructs that may be used to drive the expression of tagged PTPσ in transfected cells. In this chapter the expression of recombinant tagged PTPσ using these expression constructs is characterised. It was found that a number of PTPσ species are produced in transfected cells including full-length PTPσ and a range of fragments resulting from cleavage at specific sites. A modified nomenclature is proposed for PTPσ processing. The sub-cellular localisation of the various PTPσ species was examined using immunofluorescent and biochemical approaches. Full-length PTPσ was found to be expressed on the surface of transfected cells whereas at least a significant proportion of the cleavage products were found within the cell.

3.1: Expression of wild type PTPσ

Transfection of HEK-293T cells with vectors encoding chick PTPσ with an amino-terminal 3xFLAG tag resulted in the detection of three FLAG-reactive PTPσ species (Figure 3.1Bi). These had apparent molecular weights of approximately 160 kDa, 90kDa and 80kDa respectively. The relative level of the largest species when compared to the two smaller species varied between experiments. However, the smallest of the three species (Mw~80kDa) was always present at a higher level than the intermediately sized species (Mw~90kDa).

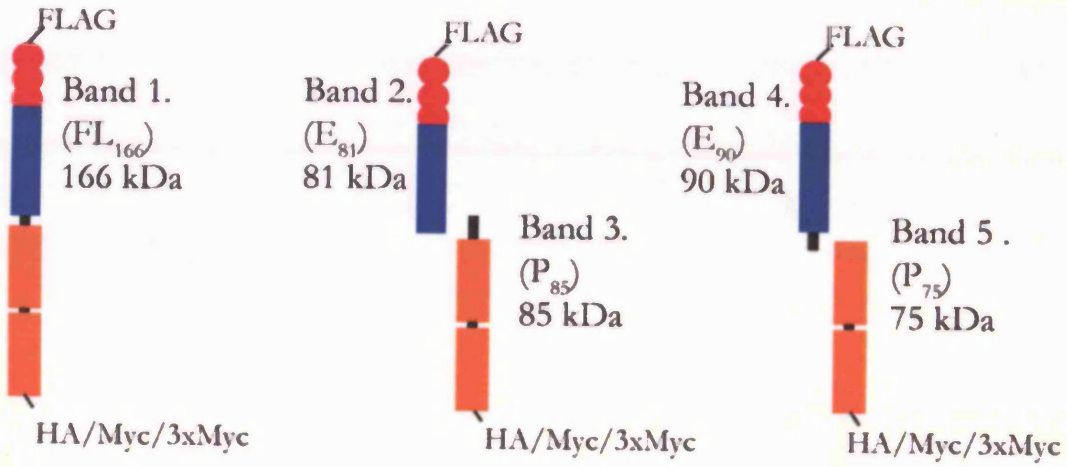
The carboxyl-terminal single Myc tag contained in the p3xFLAG-CMV25-Myc vector was not detectable in lysates in which the 3xFLAG epitope tag was successfully detected (Figure 3.1Bii). Therefore, two approaches were used to allow the detection of a carboxyl terminal epitope. Firstly, the single Myc tag was directly replaced with a single HA tag. Secondly, the single Myc tag was lengthened to a 3xMyc tag by insertion of an oligonucleotide linker. In addition, it may be noted that the single Myc-tagged protein was detectable using a different monoclonal antibody (Clone 4A6; Upstate, UK) said to be less sensitive to the tag location than the 9E10 clone (Sigma-Aldrich, UK) used in this thesis (Data not shown). In cells that overexpress appropriately tagged PTPσ, three peptide species can be detected using antibodies directed against the carboxy-terminal HA or 3xMyc tag (Figure 3.1Bii,iii). These have apparent molecular weights of approximately 160kDa, 85kDa and 75kDa respectively. In agreement with the data obtained using

antibodies directed against the amino-terminal tag, whilst the relative abundance of the largest species relative to the two smaller species varied between transfections, there was always a significant difference in the relative level of the two smaller species. In all experiments, the larger fragment (Mw~85kDa) was reproducibly more abundant than the smaller (Mw~75kDa).

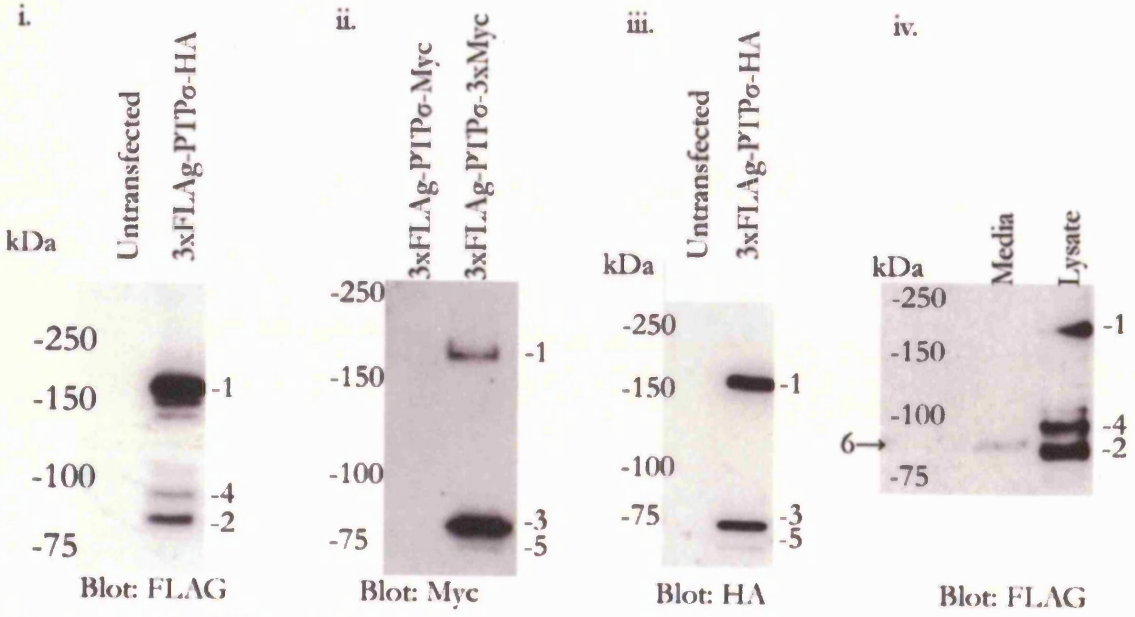
Transfection of a rat phaeochromocytoma-derived cell line (PC6-3) results in the production of the same PTPσ species that are seen in transfected HEK-293T cells (Figure 3.1C). As expected, transfection of this cell line resulted in a significantly lower transfection efficiency and a noticeably lower level of recombinant protein expression than transfection of the HEK-293T cell line (Data not shown). The extracellular and full-length species from the PC6-3 cell line appeared to run very slightly smaller than those from HEK-293T cells (Data not shown). There was no detectable difference in the size of the larger intracellular domain fragment. The smaller intracellular domain fragment was undetectable under the same conditions used to detect it in HEK-293T cells. This is likely to be due to low expression levels rather than qualitative differences in PTPσ processing.

Cells that overexpress PTPσ shed an extracellular domain fragment into the tissue culture media. The media from cells transfected with PTPσ was removed and cell debris was removed by passage through a 0.22μm filter. Analysis by SDS-PAGE and subsequent immunoblotting revealed the presence in the media of a PTPσ species that was immunoreactive for both the FLAG (Figure 3.1 B iv) and IG2 antibodies (Data not shown) (Stoker et al., 1995a). Careful examination of the apparent molecular weight of this shed species revealed that the band was different in size from both the intermediate and smallest bands seen in cell lysates (Band 2) (Figure 3.1 B). The band migrated with an apparent molecular weight very slightly larger than that of the smallest FLAG positive species (~82kDa).

A. Schematic diagram of PTPσ cleavage fragments



B. Expression of PTPσ in HEK293T cells



C. Expression of PTPσ in PC6-3 cells

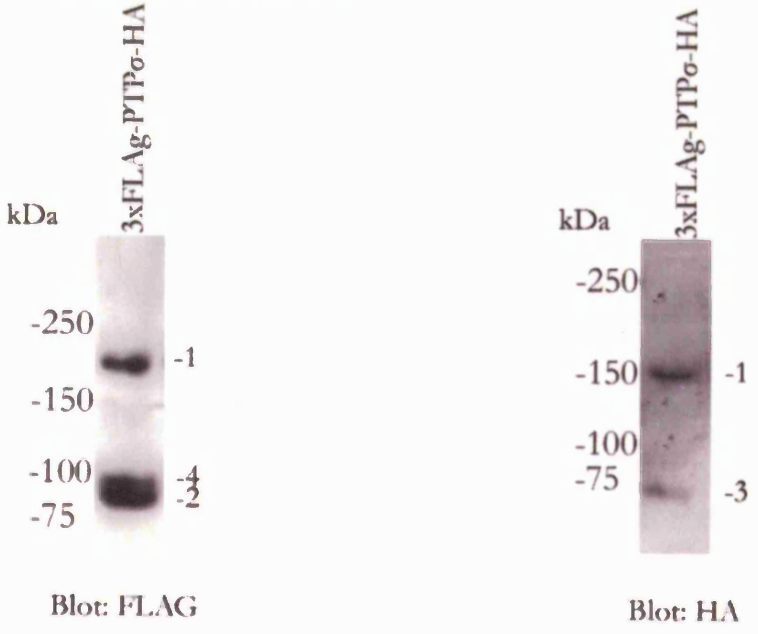


Figure 3.1: Overexpression of 3xFLAG- and HA-tagged PTPσ

A. Schematic diagram of PTPσ proteins to show tag locations and expected cleavage products.
B. Expression of 3xFLAG- and HA-tagged PTPσ in HEK 293T cells. Cells were transfected using calcium phosphate. Lysates or conditioned cell culture media (iv. lane 1) were analysed using reducing SDS-PAGE. Western blots were probed with antibody against the amino-terminal 3xFLAG tag (Panel i.), the carboxyl-terminal Myc or 3xMyc tag (Panel ii.), the carboxyl-terminal HA tag (Panel iii.) and the amino-terminal 3xFLAG tag (Panel iv.).
C. Expression of 3xFLAG- and HA-Tagged PTPσ in PC6-3 neuronal cell line. PC6-3 cells were transfected with PTPσ by J. Nixon using Effectene (Qiagen, UK). Lysates were analysed using reducing SDS-PAGE and Western blots were probed with antibody against the amino-terminal 3xFLAG tag (Left panel) or the carboxyl-terminal HA tag (Right panel).

Band 1. Full-length PTPσ 1(FL_{166})
Band 2. Extracellular domain from amino terminal cleavage (E_{81})
Band 3. Phosphatase domain from amino-terminal cleavage (P_{85})
Band 4. Extracellular domain from carboxy-terminal cleavage (E_{90})
Band 5. Phosphatase domain from carboxy-terminal cleavage (P_{75})

Previous work on chick PTPσ has utilised the IG2 antibody, which was raised against a bacterially expressed GST fusion containing the first 2 PTPσ Ig2 domains (Stoker et al., 1995a). This antibody has not previously been shown to detect full-length chick PTPσ. In addition, only a single smaller extracellular domain species has been detected rather than the two that are detectable with the FLAG antibody. The detection profile of these 2 antibodies was therefore compared. Under reducing conditions, it proved very difficult to detect overexpressed PTPσ with the IG2 antibody, despite the known high level of PTPσ in these cells (Figure 3.2 B). Indeed detection of any PTPσ expression at all under reducing conditions required significantly extended exposure times. Moreover, even under these conditions, it did not prove possible to detect full-length PTPσ and only small amounts of the intermediately sized fragment were detectable despite the abundance of both of these species in the lysate (c.f. Figure 3.1B, Figure 3.2B). Subsequent work described in this thesis makes abundant use of non-reducing SDS-PAGE. As an aside to this work, it was noted that when used to immunoblot non-reducing SDS-PAGE gels, the IG2 antibody is able to detect all three PTPσ species that contain the extracellular domain (Figure 3.2 C). However, although the relative abundance of the intermediately sized species normalises under these conditions, the relative abundance of the full-length species is still noticeably under detected.

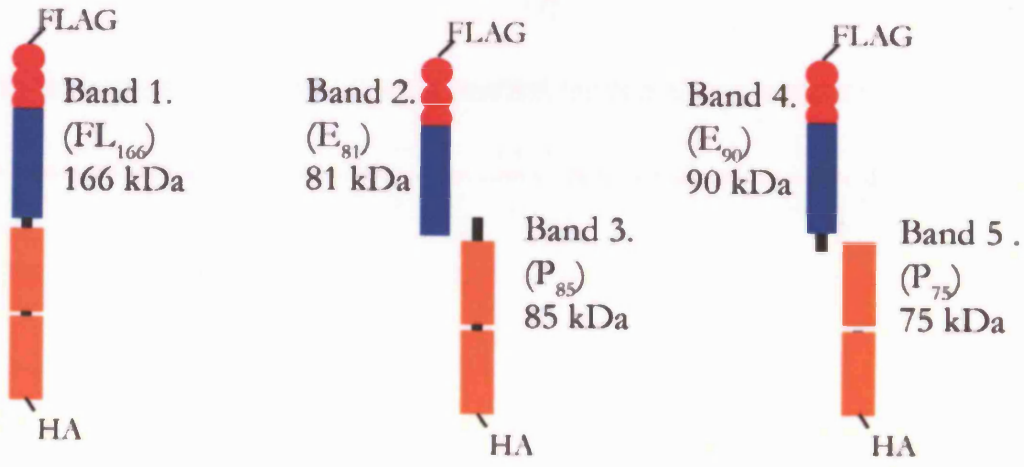
Although its interpretation is complicated, the IG2 antibody allows comparison of endogenously expressed PTPσ with that produced in HEK-293T cells. Using the IG2 antibody on lysates made from embryonic chick tissues, two species are detected with apparent molecular weights of approximately 75 and 130 kilodaltons (kDa) respectively (Figure 3.2D). These are believed to represent the extracellular fragments of the short, PTPσ1, and long, PTPσ2, isoforms respectively. However, there is no direct agreement between the size of the endogenous PTPσ1 band and the two extracellular domain fragments as expressed in HEK-293T cells. The endogenous band is slightly smaller than those seen when PTPσ is overexpressed (Figure 3.2D). However, the difference is larger than that which would be expected from the inclusion of the 3xFLAG tag.

It is possible that the reduced size of the extracellular domain fragments seen when PTPσ was expressed endogenously or in neuronal cells reflects a difference in post-translational modification between these different types of cell. The magnitude of the difference is in the order of 5-10kDa. A possible mechanism for this difference might be differential glycosylation. Indeed it is likely that PTPσ is glycosylated on several residues (Stoker, 1994). Therefore, PTPσ overexpressed in HEK-293T cells was digested with

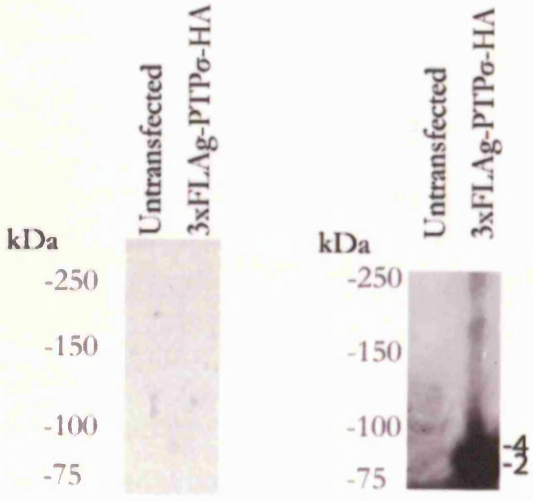
PNGase F, which removes N-linked sugar residues from asparagine residues in the peptide backbone. This resulted in a small decrease in the molecular weight of all PTPσ species containing extracellular domain constituents (Figure 3.2E). Following digestion with PNGase F, the smallest PTPσ extracellular domain species migrated with an apparent molecular weight approximately 10kDa less than following mock digestion. Similarly, the intermediate fragment migrated approximately 5-8kDa faster. It was difficult to determine whether the full-length protein also migrated faster following PNGase F treatment and there was no discernable effect on intracellular domain fragment size (Data not shown). The effect of degylycosylation on PTPσ size suggested that it does not exhibit extensive glycosylation. This is in keeping with the small number of predicted glycosylation sites (1.2.1).

It was noted above that the proportion of PTPσ found in the full-length or cleaved form varied significantly between experiments. Experience suggested that some of this variability was due to the amount of time between transfection and cell harvesting. Harvesting cells at set time-points post-transfection showed that the appearance of PTPσ species followed a predictable pattern (Figure 3.3C). FL_{166} appeared first, at approximately 16 hours post transfection. In contrast, E_{90} and E_{81} were only detectable from approximately 32 hours post-transfection. Moreover, E_{81} appeared earlier than E_{90}. It might be suggested that the observed effects of time post-transfection on cleavage pattern might reflect differences in the relative exposure of lanes containing little PTPσ and lanes containing large amount of PTPσ. However, comparison of equivalently exposed lanes (Figure 3.3C Panel i lane 4; panel iii lane 5) reveals that this is not the case. The pattern of PTPσ cleavage therefore appears to change during the course of this experiment. One possible explanation for this difference is that the HEK-293T cells continued to proliferate throughout the experiment increasing cell density, another might be that the transfection method inhibits protein expression and that this effect diminished with time. However, as the cleavage pattern of RPTPs may be affected by cell density (Campan et al., 1996), a complimentary experiment was designed to determine the effects of cell density on PTPσ expression level and cleavage pattern.

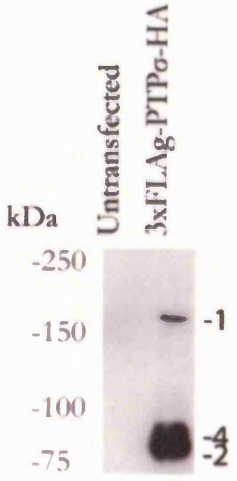
A. Schematic diagram of PTPσ cleavage fragments



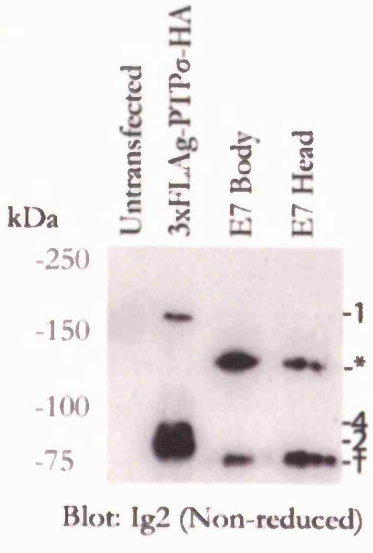
B. Ig2 detection - reducing SDS-PAGE



C. Ig2 detection - non-Reducing SDS-PAGE



D. Endogenous PTPσ expression



E. Glycosylation status of PTPσ peptides

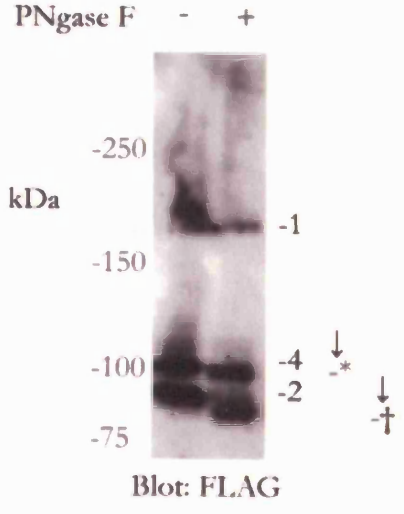


Figure 3.2: Analysis of IG2 antibody detection pattern and PTPσ glycosylation

A. Schematic diagram of PTPσ proteins to show tag locations and expected cleavage products.

B., C. Detection of overexpressed 3xFLAG-PTPσ-HE using IG2 antibody under reducing (B) and non-reducing (C) conditions using the same lysate shown in Figure 3.1 B-C. Samples were analysed in parallel with identical exposure times (Standard exposure = 1 minute; long exposure = 1 hour).

D. Non-reducing detection of endogenous PTPσ expression in lysates from embryonic chick tissue.

E. Glycosylation status of PTPσ peptides. HEK-293T cells were transfected with 3xFLAG-PTPσ-HA. Lysates were treated with PNGase F (+) or mock digested (-). The effects of PNGase F digestion were analysed by SDS-PAGE and western immunoblotting using antibodies against the amino-terminal 3xFLAG tag

Band 1. Full-length PTPσ 1 (FL_{166})
Band 2. Extracellular domain from amino terminal cleavage (E_{81})
Band 3. Phosphatase domain from amino-terminal cleavage (P_{85})
Band 4. Extracellular domain from carboxy-terminal cleavage (E_{90})
Band 5. Phosphatase domain from carboxy-terminal cleavage (P_{75})

* Cleaved extracellular domain from PTPσ2

† Cleaved extracellular domain from PTPσ1

* Deglycosylated band 4

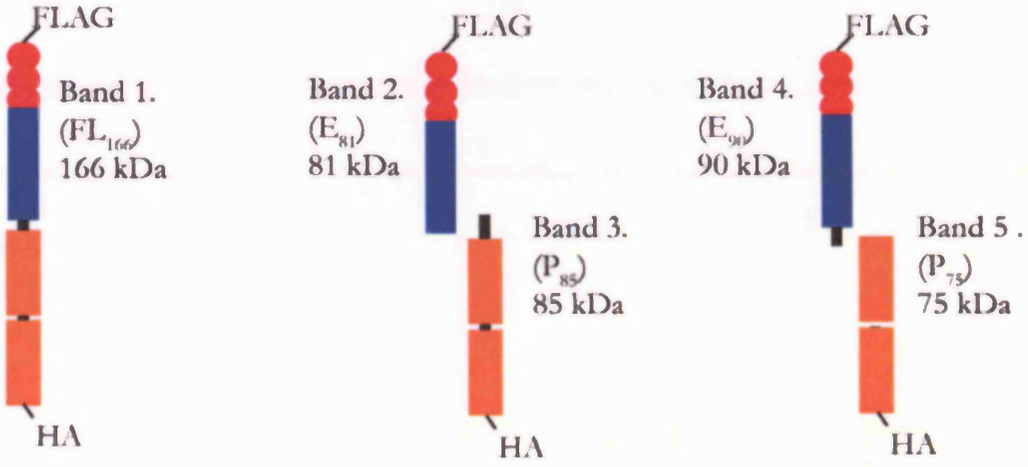
† Deglycosylated band 2

Cells at a range of cell densities were harvested 64 hours after transfection, representing the final time point in the time-course experiment, at which all three FLAG-positive species were detectable. At low cell densities only FL_{166} was detectable whereas only above normal cell densities ($2.5x10^5$ cells/ml) were all three species detectable (Figure 3.3B). However, comparison of equivalently exposed lanes suggested that the apparent effect of cell density on PTPσ cleavage may be due to the relative exposure of each lane (Figure 3.3B Panel i lane 4; panel iii lane 6). It must be noted that in addition to cellular proliferation, the HEK-293T cell line would also exhibit plasmid amplification . It is not clear whether the degree of plasmid amplification was consistent in cells at all densities. Finally, in the time-course and cell density experiments, the total amount of PTPσ increased in proportion to increasing time post-transfection or increasing cell density, respectively. Equal amounts of total protein (Bradford Assay) were loaded into each lane. However, it is clear from the Brilliant blue stained gels (Figure 3.3 B panel ii, C panel ii) that wells contained variable amounts of lysate and that this was balanced by differences in the contribution of bovine serum albumin (BSA) to the sample. Despite these problems however, the observed differences in total protein level (at most 5-fold) appeared not to account for all the increase in PTPσ levels (>10-fold).

3.2: Sub-cellular localisation of PTPσ

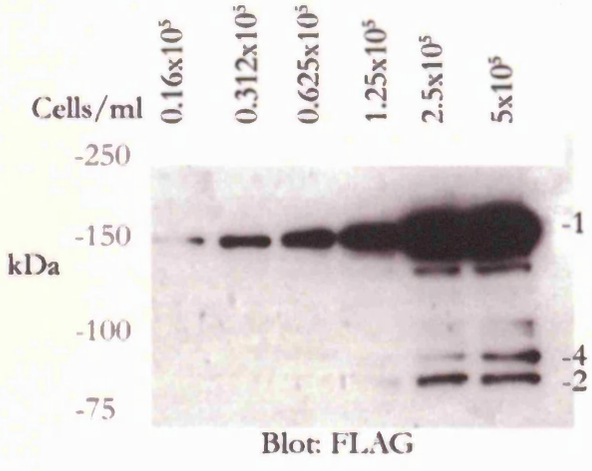
The finding that both full-length and cleaved PTPσ could be detected using SDS-PAGE raised the concern that the full-length PTPσ was an artefact. This might be due to the accumulation of overexpressed protein that is not appropriately modified by the PTPσ cleavage apparatus. For example, this might reflect the formation of insoluble aggregates containing overexpressed protein or the saturation of cellular processing systems by overexpressed protein resulting in the upstream accumulation of immature full-length PTPσ. Under this second model, the additional intermediate band detected might represent an "escape" processing pathway utilised only when PTPσ levels exceed that which can be contained within the normal processing pathways. Therefore, the localisation of PTPσ within transfected cells was assessed using two different techniques. Firstly, the localisation of PTPσ in fixed cells was assessed using immunofluorescence and then the accessibility of PTPσ to extracellular trypsin was assessed using living PTPσ-transfected cells.

A. Schematic diagram of PTPσ cleavage fragments



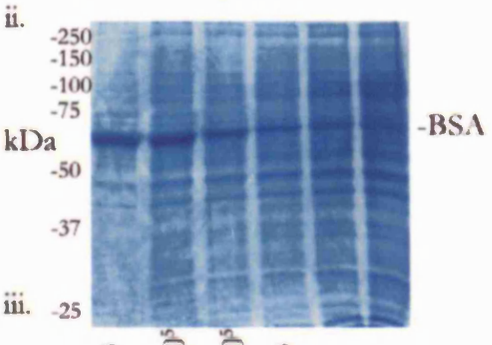
B. Effect of cell density on cleavage pattern

i.

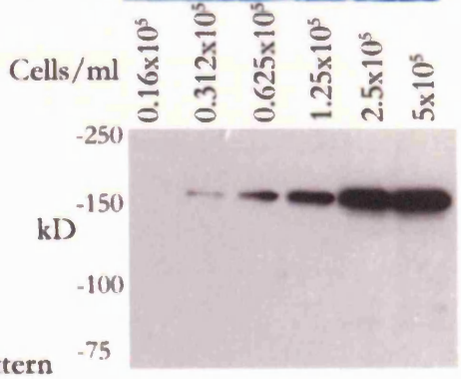


Blot: FLAG

ii.

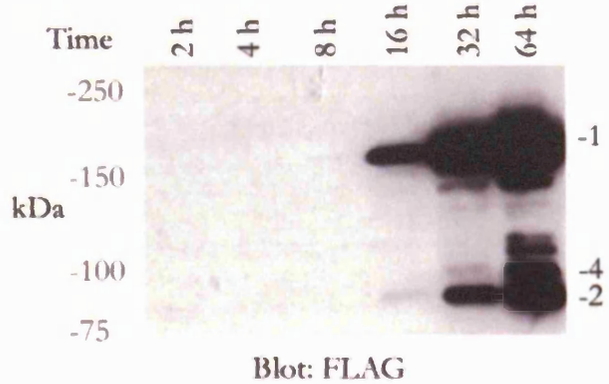


iii.



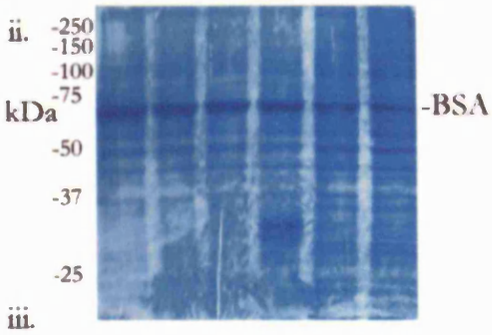
C. Effect of time post transfection on cleavage pattern

i.



Blot: FLAG

ii.



iii.

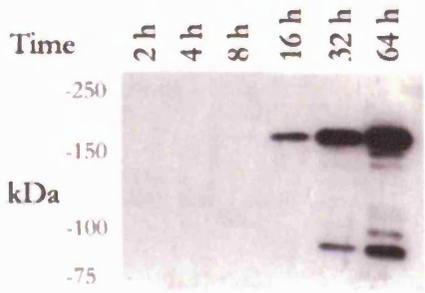


Figure 3.3: Effects of cell density and duration since transfection on PTPσ cleavage pattern

A. Schematic diagram of PTPσ proteins to show tag locations and expected cleavage products.
B. Effect of increasing cell density on PTPσ cleavage pattern. HEK-293T cells were transfected with PTPσ and then split into plates at a range of cell densities. Cells were harvested 64 hours after transfection and the PTPσ cleavage pattern was analysed by SDS-PAGE and western immunoblotting using antibodies against the amino-terminal 3xFLAG tag(Panel i). A shorter exposure is also shown to allow comparison of equivalent exposures (Panel iii). Gels were also analysed by Brilliant Blue staining to show total protein content (Panel ii).
C. Effect of increasing time since transfection on PTPσ cleavage pattern. HEK-293T cells were transfected and then allowed to express PTPσ for periods of time ranging from 2 hours to 64 hours after removal of the transfection mix. Cells were harvested and the PTPσ cleavage pattern was analysed by SDS-PAGE and western immunoblotting using antibodies against the amino-terminal 3xFLAG tag (Panel i). A shorter exposure is also shown to allow comparison of equivalent exposures (Panel iii). Gels were also analysed by Brilliant Blue staining to show total protein content (Panel ii).

10μg total protein was loaded for each sample.

Band 1. Full-length PTPσ 1(FL_{166})
Band 2. Extracellular domain from amino terminal cleavage (E_{81})
Band 3. Phosphatase domain from amino-terminal cleavage (P_{85})
Band 4. Extracellular domain from carboxy-terminal cleavage (E_{90})
Band 5. Phosphatase domain from carboxy-terminal cleavage (P_{75})

BSA Bovine serum albumin from the cell culture media

3.2.1: Immunofluorescent analysis of wild type PTPσ protein

Under the conditions used, neither the anti-FLAG nor the anti-HA antibody exhibited significant levels of background staining on untransfected cells (Figure 3.4B, C). In addition, there was no significant background seen when cells were detected with control serum and specific secondary antibodies (Data not shown).

Using the anti-FLAG antibody, which detects the amino-terminal tag, cell surface labelling was observed (Figure 3.4E, Arrows Figure 3.5D, G). In addition, in some cells a filamentous pattern of fluorescence was seen in association with the presence of discrete subcellular rings of signal (Arrow and arrowheads Figure 3.5A). This pattern may represent an optical section including cell-matrix adhesions at the cell base. There was evidence of an increase in signal intensity in areas of cell-cell contact (Figure 3.5G). However, this effect was not uniformly seen and was not clearly associated with contact either between two PTPσ expressing cells or between one cell expressing PTPσ and one that did not. Finally in some cells, FLAG-positive processes were seen (Figure 3.5K-M).

Immunofluorescent detection of the carboxy-terminal HA tag produced a similar picture of membrane localisation, although filamentous staining was not seen and the ring staining seen with the FLAG antibody was less clear using the HA antibody (Figure 3.4F, Figure 3.5B, E, H). However, two additional staining patterns were seen with the HA antibody. Firstly, the apparent depth of the pericellular staining was greater using the carboxyl-terminal HA tag than that seen using the amino-terminal FLAG tag. Secondly, in some cells a ring of perinuclear signal was seen (Figure 3.5B, C – Open arrowheads).

Staining using the two tags generally agreed as can be seen by artificially overlaying the FLAG signal (green) over the HA signal (red). Areas of overlap were seen by the merge colour, yellow (Figure 3.4G, Figure 3.5C, F, I). However, as previously noted, the HA signal forms a less distinct perinuclear ring than the FLAG signal.

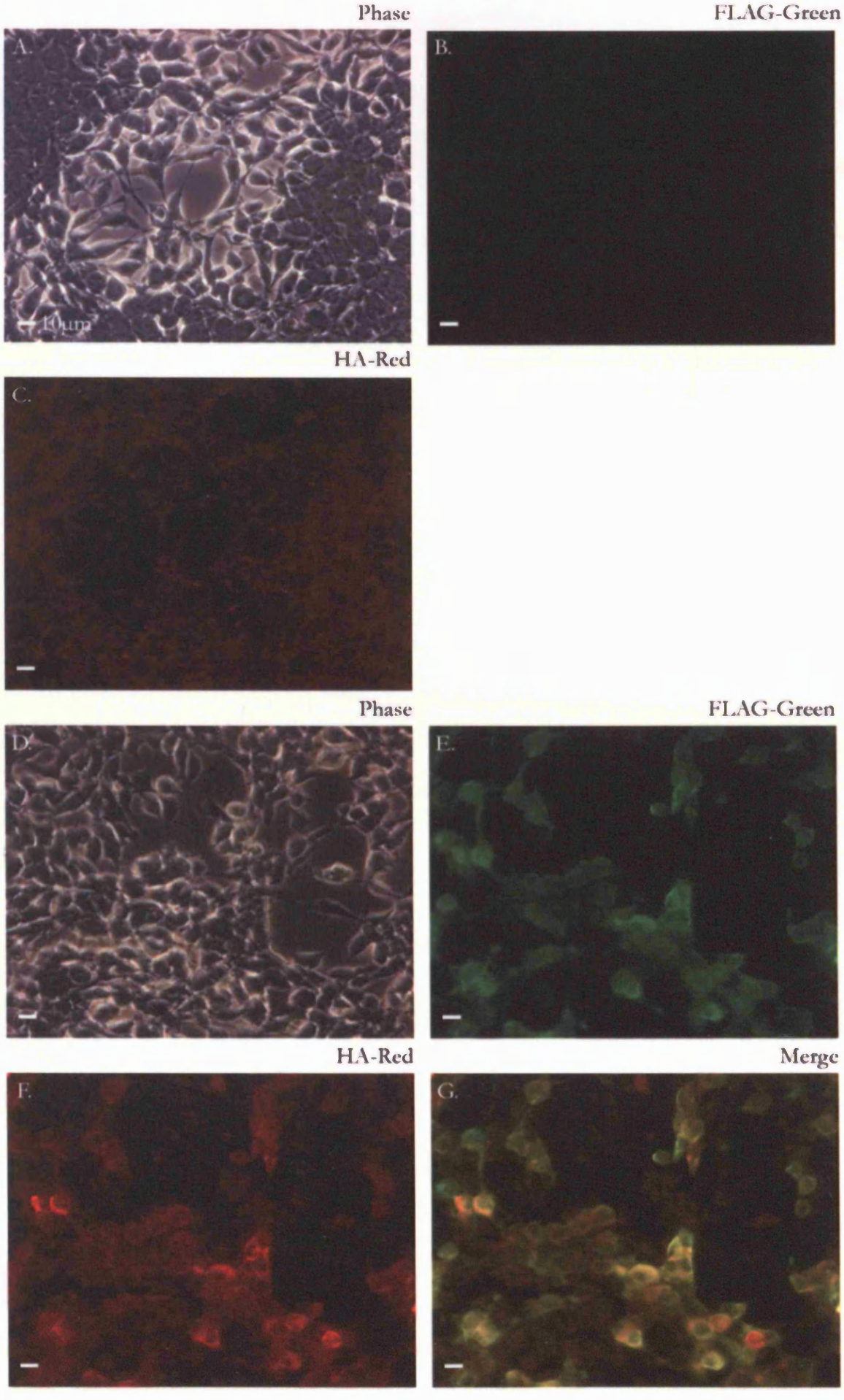

Phase
FLAG-Green
A.
10μm
B.
HA-Red
C.
Phase
FLAG-Green
D.
E.
HA-Red
Merge
F.
G.

Figure 3.4: Immunofluorescent localisation of overexpressed PTPσ – 1

A-C. Control untransfected cells.

A – Phase contrast; B – anti-FLAG detection; C – anti-HA detection

D-G Wild type PTPσ-transfected cells,

D – Phase contrast; E – anti-FLAG detection; F – anti-HA detection; G – Merge of HA and FLAG signal

HEK 293T cells were grown on polyethyleneimine-coated coverslips and either mock-transfected or transfected with wild type PTPσ using the calcium phosphate method. Cells were washed and fixed 72 hours after transfection. The PTPσ construct used contained an amino-terminal FLAG tag, and a carboxyl-terminal HA tag. The FLAG epitope was detected with FITC-labelled secondary antibody (green) and the HA epitope was detected with Cy3-labelled secondary antibody (red). Scale bars (10μm) are included in each image. All images were obtained at 400x magnification using Axiovision software on an Axioskop II microscope (Zeiss).

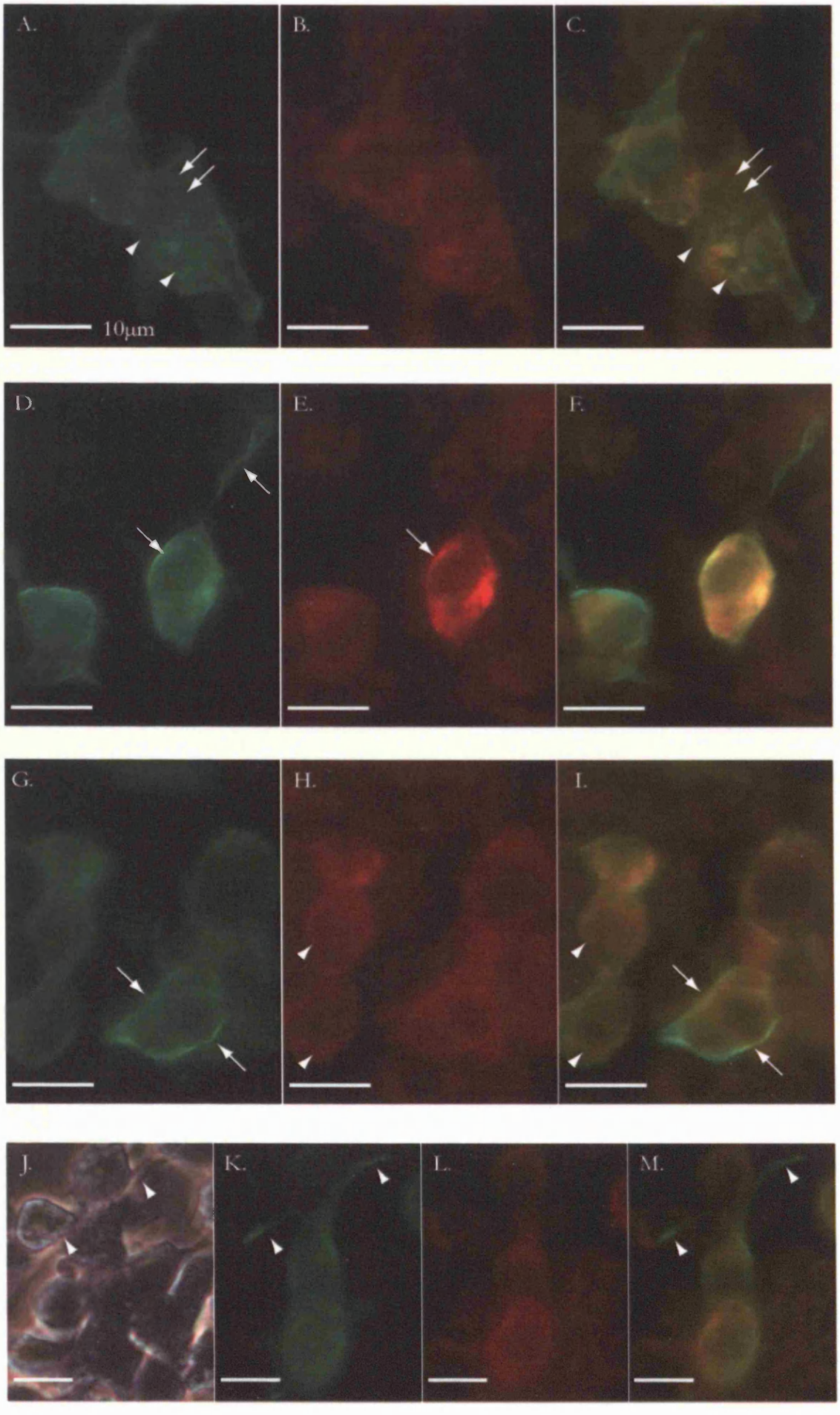

A.
B.
C.
10μm
D.
E.
F.
G.
H.
I.
J.
K.
L.
M.

Figure 3.5: Immunofluorescent localisation of overexpressed PTPσ – 2

HEK 293T cells were grown on polyethyleneimine-coated coverslips and transfected using the calcium phosphate method with wild type PTPσ bearing an amino-terminal FLAG tag and a carboxyl-terminal HA tag. The FLAG epitope was detected with FITC-labelled secondary antibody (A, D, G – green) and the HA epitope was detected with Cy3-labelled secondary antibody (B, E, H – red). Scale bars (10μm) are included in each image. All images were obtained at 400x magnification and merged images (C, F, I) were produced using Axiovision LE software (Rel. 4.5) on an Axioskop II microscope (Carl Zeiss).

A-C: Arrowheads indicate filamentous intracellular FLAG signal. Arrows indicate oval bodies surrounded by FLAG signal

D-F: Arrows indicate cell membrane signal

G-I: Arrows indicate membrane-localised FLAG signal, arrowheads indicate perinuclear HA signal

J-M: Arrow heads indicate cytoplasmic process with FLAG signal

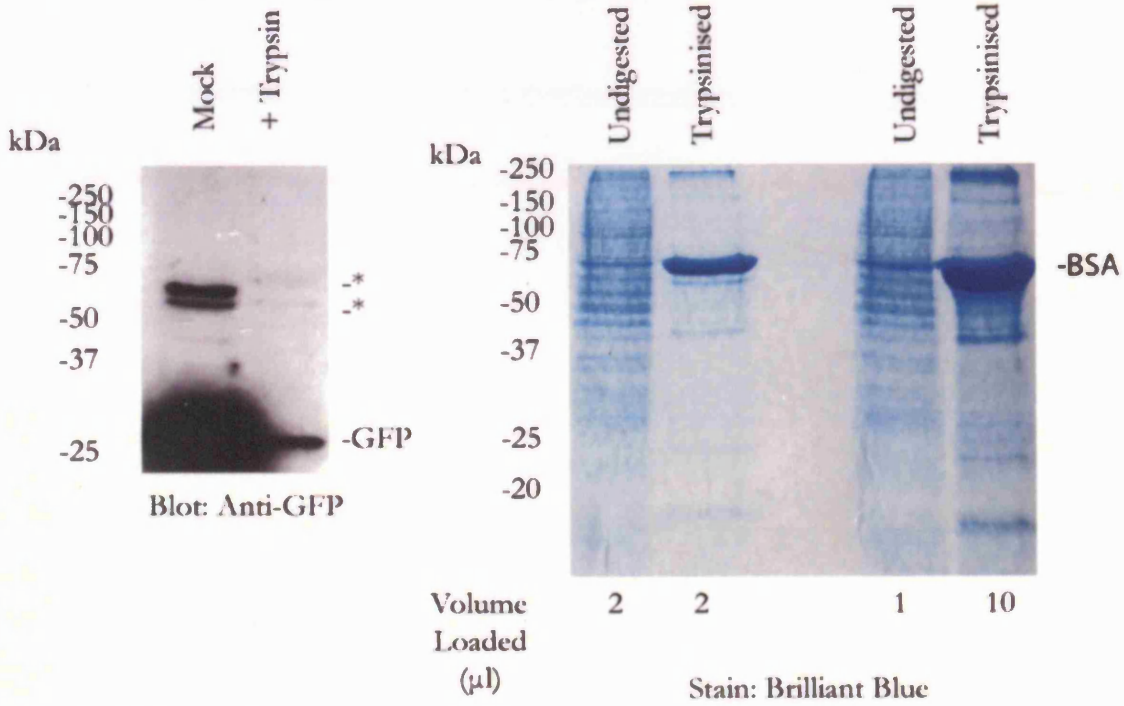
3.2.2: Analysis of cell surface exposure of PTPσ peptides by limited trypsin digestion

The presence of PTPσ protein at the cell surface was also assessed using limited trypsin digestion, which exposes living cells expressing the protein of interest to a dilute solution of trypsin. Theoretically, only peptide chains outside the protection of the plasma membrane are sensitive to digestion. Intracellular peptide chains are relatively protected from digestion.

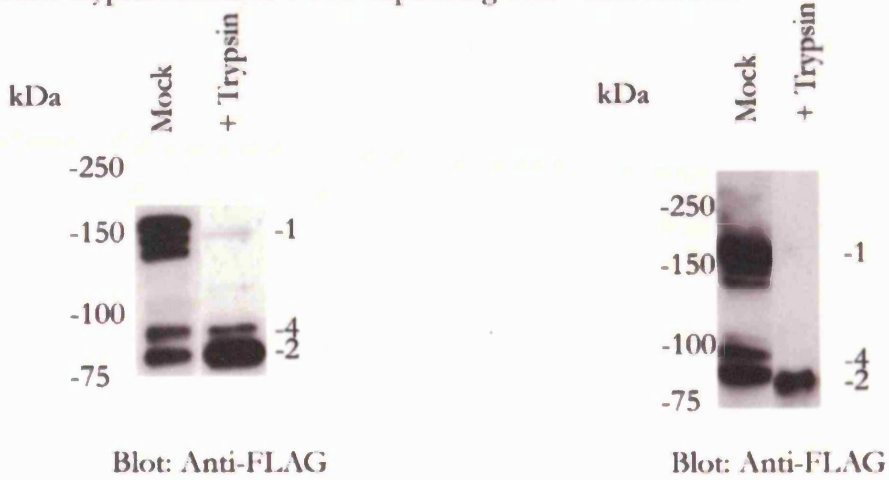
Control experiments using the intracellular green fluorescent protein were initially discouraging as this intracellular protein was subject to significant amounts of digestion. This can be seen both from anti-GFP immunoblotting and from comparison of the total protein content of treated and untreated lysates using Brilliant Blue staining (Figure 3.6A). This was due to loss of cells following trypsinisation. Unfortunately, HEK-293T cells are easily detached form culture-ware even in the absence of trypsin. However, experience of the technique resulted in the ability to trypsinise cells under conditions where only a minority of cells were lost and so the total protein content was not significantly affected (Figure 3.6C). Moreover as exemplified by the results with PTPσ, under these conditions, specific bands were either unaffected or disappeared entirely following trypsinisation.

Limited cell surface trypsinisation of PTPσ-transfected cells resulted in the specific disappearance of full-length PTPσ, with the specific preservation of the level of the smallest PTPσ species detected by the FLAG antibody (Figure 3.6B). The low level of the intermediately sized fragment in many transfections made it difficult to form a clear picture of the effect of trypsinisation on this species. However, in some experiments it appeared to be unaffected by digests that completely removed full length PTPσ and so it is likely that this band was also relatively protected from trypsin when compared to the full-length protein.

A. Control cell surface trypsinisation



B. Cell surface trypsinisation of PTPσ expressing cells - immunoblot



C. Cell surface trypsinisation of PTPσ-expressing cells - Brilliant blue stain

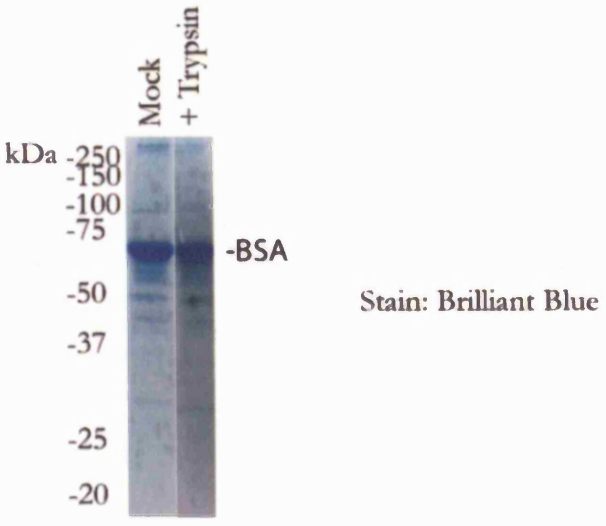


Figure 3.6: Subcellular localisation of PTPσ using limited cell surface trypsinisation

A. Control cell surface trypsinisation. Cells transfected with green fluorescent protein (GFP) were digested with trypsin. The control sample represents a lysate of cells that were not treated in any way. Lysates were analysed by reducing SDS-PAGE and western immunoblotting using an antibody against GFP. SDS-PAGE gels were also analysed by brilliant blue staining to demonstrate the reduction in total protein content. The BSA band resulting from the trypsin inhibitor solution is clearly visible in digested samples.
B. Cell surface trypsinisation of PTPσ expressing cells. Anti-FLAG immunoblots. Two independent experiments are shown. Mock and +trypsin samples were prepared in parallel; the mock sample digestion solution lacked trypsin only.
C. Cell surface trypsinisation of PTPσ-expressing cells. Total protein detected using brilliant blue stain. Equal volumes of lysate were used; samples were not normalised for protein content. The lanes shown represent non-adjacent samples on the same gel. The gel was processed intact and the lanes subsequently excised for ease of comparison. The BSA band produced by the trypsin inhibitor solution is clearly visible as are several bands from the lysates themselves

3.3: Discussion

This chapter introduces the PTPσ expression constructs that will be used throughout the remainder of this thesis. Although the expression of chicken PTPσ has previously been described using antibodies to the extracellular domain (Stoker et al., 1995a), this represents the first description of chicken PTPσ expression using a combination of antibodies recognising both the extracellular and the intracellular domains. The findings presented confirm previous work on rat and human PTPσ (Aicher et al., 1997). On SDS-PAGE analysis, lysates from PTPσ-expressing cells contained a number of PTPσ species that represented full-length PTPσ and several PTPσ-derived cleavage fragments. To simplify the discussion of PTPσ processing, a modified nomenclature will be proposed for PTPσ processing and the resulting PTPσ cleavage fragments. The identity of each species seen on SDS-PAGE analysis was predicted with reference to previous studies on type II RPTPs. Of the PTPσ-derived species reported here, full-length chicken PTPσ was present at higher than expected levels and a novel extracellular cleavage fragment (E_{90}) was described. The discussion of these findings raises several issues that are of significance in the field. Several lines of evidence are presented that suggest that the current model of type IIa RPTP processing is incomplete.

The post-translational processing of type IIa RPTPs is complicated, generating at least five PTPσ peptides from a combination of at least two cleavage events. In this thesis, it will be necessary to discuss the various PTPσ species and varying combinations of cleavage events in an unambiguous manner. An extension of the existing nomenclature is suggested to allow the unambiguous discussion of both previously described species and of novel species. (Yu et al., 1992; Streuli et al., 1992). Conventionally, the two post-translational cleavage events affecting type IIa RPTPs are referred to as primary and secondary cleavage, acknowledging their perceived temporal relationship. However, this thesis will show that secondary cleavage can occur independently of primary cleavage. Therefore, it is clearer to refer to these two cleavage events by their spatial location, that is amino- (Primary) and carboxyl-terminal (Secondary) cleavage. Secondly, previous studies have referred to single extracellular (E) and phosphatase (P) fragments generated by post-translational cleavage (Streuli et al., 1992; Aicher et al., 1997). In this thesis, a number of PTPσ cleavage fragments will be introduced. To distinguish between species it is proposed that their approximate molecular weight be added to their E/P fragment designation. Thus, full-length protein will be referred to as FL_{166} and the extracellular domain products of amino- and carboxyl-terminal cleavage as E_{81} and E_{90} respectively. In the absence of

confirmation that the shed extracellular domain form is identical to either of the cell associated forms, shed protein will be referred to as E_s. The phosphatase domain fragments resulting from amino- and carboxyl-terminal cleavage will be referred to as P_{85} and P_{75}, respectively. This nomenclature was summarised in pictorial form above (Figure 3.1A) and will be used for the remainder of this thesis.

PTPσ-expressing HEK-293T cell lysates contain IG2 /FLAG/HA positive PTPσ-derived species with apparent molecular weights of ~160/90/80 kDa (FLAG/IG2 detection) and ~160/85/75 (HA detection) (Figure 3.1, Figure 3.2)(Stoker et al., 1995a). The ~160 kDa band, which is recognised by all 3 antibodies, is likely to represent full-length PTPσ (Predicted Mr~165.7 kDa. Regarding the smaller species, type II RPTPs undergo proteolytic cleavage at several sites (reviewed above, 1.3.5). Based upon previous studies, the ~80 kDa FLAG/IG2-positive, and the ~85 and ~75 kDa HA-positive species are likely to represent the extracellular domain product of amino-terminal cleavage (E_{81}) and the intracellular domain products of amino- (P_{85}) and carboxyl-terminal (P_{75}) cleavage, respectively (Streuli et al., 1992; Pulido et al., 1995a; Aicher et al., 1997). Although previously unrecognised, carboxyl-terminal cleavage occurring in the absence of amino-terminal cleavage would generate P_{75} and an extracellular domain fragment with the same characteristics as the remaining ~90kDa FLAG/IG2 detectable species (E_{90}). It must be noted that the presence of the E_{90} species in cell lysates implies that carboxyl-terminal cleavage is not sufficient for ectodomain shedding. Previous studies have shown that amino-terminal cleavage is not necessary for ectodomain shedding (Serra-Pages et al., 1994). The presence of the E_{90} and significant amounts of FL_{166} were unexpected from previous studies, which had predicted that lysates would contain mainly E_{81} and P_{85} (Streuli et al., 1992; Stoker et al., 1995a; Aicher et al., 1997). Each of these two unusual findings will be explored in more detail below.

PTPσ-expressing cells also secrete a soluble form of the PTPσ extracellular domain (E_s) (Figure 3.1B) (Aicher et al., 1997). The identity of the shed band and the mechanism and purpose of type IIa PTPσ extracellular domain shedding are not fully understood (Serra-Pages et al., 1994; Aicher et al., 1997). Studies of chick PTPσ in tissue and transfected cells have reported only a single extracellular domain species in cell lysates (Stoker et al., 1995a). In contrast, chick tissue lysates contain 2 extracellular domain species, that represent the cleaved extracellular domains of short (PTPσ1) and long (PTPσ2) PTPσ isoforms (Stoker et al., 1995a; Stoker et al., 1995b). It is thought that this single PTPσ1-derived extracellular domain species seen in tissue and cell lysates represents the PTPσ1 E_{81}

molecule. A case may be made from the literature that this species might represent the E_{81} or E_{90} species or an accumulation of cell bound E_s (Aicher et al., 1997; Krasnoperov et al., 2002). However, functional interactions have been demonstrated for both E_{90} and E_{81} forms, suggesting that they are both physiological forms of the protein (Krasnoperov et al., 2002). In addition, the level of cell-associated E_{90} is of the same order of magnitude as the E_{81} band, implying that the majority would have to bind back onto an unknown receptor on the cell surface. As will be shown later, whilst cell-binding experiments suggest that PTPσ extracellular domains can bind to HEK-293T cells, they do not support an effect of this magnitude (7.1.2).

In this study, SDS-PAGE analysis revealed that E_S was slightly different in size from both the ~90kDa and the ~80kDa FLAG-positive species and also from PTPσ species seen in the developing chick. Unfortunately, this study is one of very few to compare directly the size of shed ectodomain with those of the cell-associated proteins and in previous studies it can be difficult to identify single PTPσ cleavage fragments in lysates due to the small size difference between E_{81} and E_{90} and the presence of multiple PTPσ isoforms. Furthermore, previous studies have not shown a consistent relationship between the sizes of the various forms of type IIa RPTPs and some studies report shedding of both the large and small extracellular domain fragments (Serra-Pages et al., 1994; Pulido et al., 1995a). There are several possible explanations for the differences between the sizes of E_S and other PTPσ species reported here and elsewhere. Firstly, the difference seen may reflect differences in buffer conditions. Alternatively, it is possible that there is a real difference in size between these species. This may be due to differences in post-translational modification or indeed cleavage at different sites. Cleavage at a novel site might be mediated by a different enzyme as has recently been described in PTPκ and PTPμ (Anders et al., 2006) or may reflect the sliding specificity of the enzyme responsible for classical carboxyl-terminal cleavage; it is able to recognise any of a number of overlapping cleavage sites (Serra-Pages et al., 1994). Whatever the explanation, these data suggest that caution must be exercised in identifying PTPσ cleavage fragments and in interpreting data comparing PTPσ from different expression systems. This is especially true of any conclusions that may be drawn regarding PTPσ function. PTPσ expressed in HEK-293T cells may not be behaving in the same way as endogenously expressed PTPσ.

This chapter represents the first report of full-length chicken PTPσ in cell lysates, although full-length forms of PTPσ and other type II RPTPs have previously been reported in other species (Streuli et al., 1992; Mizuno et al., 1993; Brady-Kalnay and Tonks,

1994; Rotin et al., 1994; Stoker et al., 1995a; Stoker et al., 1995b; Kypta et al., 1996; Fuchs et al., 1996; Cheng et al., 1997; Krasnoperov et al., 2002; Ostenson et al., 2002; Ruhe et al., 2006; Lajus and Lang, 2006). In many but not all previous studies, full-length PTPσ has only been reported in lysates from transfected and therefore PTP-overexpressing cells (Figure 3.1)(Streuli et al., 1992; Serra-Pages et al., 1994; Aicher et al., 1997; Krasnoperov et al., 2002) whereas lysates from tissues that express PTPσ endogenously often only contain the cleaved form of the receptor (Stoker et al., 1995a). However, it was shown above that on reducing SDS-PAGE analysis, only cleaved protein is detectable with the IG2 antibody using standard detection conditions. In contrast, on non-reducing SDS-PAGE analysis, the overall signal obtained is significantly greater and full-length PTPσ can be detected, although its level still appears to be reduced relative to cleaved protein when compared to the picture seen with the FLAG antibody (Figure 3.1, Figure 3.2). Therefore, in comparison with the epitope tag antibodies used in this thesis, the IG2 antibody appears preferentially to recognise cleaved rather than full-length protein. Although the mechanism for such preferences is unclear, they have been previously reported for type IIa RPTP antibodies (Aicher et al., 1997; note lack of full length form in immunoblots using extracellular domain antibodies compared to immunoprecipitates made with carboxyl-terminal antibody). As previous studies on chick PTPσ have used the IG2 and RP1 antibodies it is possible that tissue and transfected QT6 lysates might contain other PTPσ species that remain undetectable with the IG2 antibody (Stoker et al., 1995a; Stoker et al., 1995b). However, analysis of tissue lysates under non-reducing conditions failed to identify any full-length PTPσ (Figure 3.2), although the overall detection level was still low. Therefore, although previous studies may not have been equipped to detect full-length PTPσ for technical reasons, it appears that there may be genuine differences between this and previous studies.

The hypotheses used to explain the presence of full length PTPσ in some studies might essentially be reduced to the proposition that some expression systems lack adequate levels of the appropriate enzyme pathways to process PTPσ in the normal manner. Therefore, it has been argued that the detection of full length protein represents an artefactual accumulation of immature protein (Streuli et al., 1992; Aicher et al., 1997). Based on a careful re-examination of previous studies and using the data presented in this chapter it will be argued below that full-length PTPσ is not an artefact of overexpression but rather a mature cell surface receptor. It will be shown that there are several possible reasons for the discrepancy between studies that do not demonstrate full-length PTPσ and

those that do. Firstly, not all cells transfected with type IIa RPTPs produce detectable levels of full-length protein (Stoker et al., 1995b). This is not solely down to the reduced expression level obtained in some expression systems as PTPσ-transfected PC6-3 neuronal cells showed a similar pattern to that seen in transfected HEK-293T cells (Figure 3.1) despite expressing significantly lower levels of PTPσ (Data not shown). Although it is not known whether PC6-3 cells express PTPσ, their parental cell line (PC12) does and therefore it is likely that PC6-3 cells possess the requisite processing machinery (Wagner et al., 1994; O'Grady et al., 1994; Lajus and Lang, 2006). Secondly, in time course studies (Figure 3.3C), full-length PTPσ was the first form to appear. There was no detectable period of low expression preceding this during which PTPσ was cleaved in a "physiological" manner. However, "physiological" cleavage may have occurred at PTPσ levels below the detection threshold of this assay. Finally and perhaps conclusively, not only do untransfected PC12 and insulinoma cell lines express detectable levels of full-length PTPσ, full-length PTPσ can be detected in pancreatic tissue lysates from wild type animals (Ostenson et al., 2002; Lajus and Lang, 2006). It may be stated that the presence of full-length type II RPTPs in tissue lysates from wild type animals precludes the notion that this form is simply an artefact of overexpression systems. Therefore, the key issue is not the presence of full-length protein in some studies, but rather what affects the cleavage of type IIa RPTPs.

Although the presence of full-length PTPσ is not simply an artefact of overexpression, it might not reflect the mature functional form of the receptor (Streuli et al., 1992). Unfortunately, our understanding of type IIa RPTP function does not currently allow us to determine which receptor forms are competent to bind ligand and respond appropriately. However, several features of a transmembrane receptor may be absent in immature proteins including mature glycosylation and cell surface localisation.

In this study, both extracellular PTPσ cleavage fragments were glycosylated but the resolution of the SDS-PAGE system used made it difficult to form any conclusion regarding the glycosylation state of full-length PTPσ (Figure 3.2). However, previous studies have concluded that the full-length form of type II RPTPs is glycosylated although its glycosylation state may be less mature than that of the cleaved form (Yu et al., 1992; Streuli et al., 1992). A number of experimental approaches might be used to refine this question.

Immunofluorescence studies showed a predominantly cell surface pattern of PTPσ localisation (Figure 3.4, Figure 3.5). In addition, it was noted that a number of PTPσ-expressing cells appeared to be undergoing detachment and possibly entering apoptosis. Previous studies have suggested a role for LAR and PTPμ in programmed cell death (Weng et al., 1998; Weng et al., 1999; Tisi et al., 2000; Ensslen et al., 2003). Detailed studies of cell death were not performed during this thesis but remain an interesting avenue to explore as cell death might be a factor in the difficulties encountered in establishing stable transfectants (Unpublished observations, Dr A. Stoker; stable PTPσ transfectants have been previously reported (Suarez et al., 1999)). The finding that PTPσ is predominantly membrane-localised despite the significant proportion of uncleaved protein agreed with previous studies showing that mutations, which block PTPσ cleavage, do not affect surface expression (Serra-Pages et al., 1994). Unfortunately, it was not technically possible to distinguish between full length PTPσ and the PTPσ cleavage products using immunofluorescence. However, given the significant proportion of the total amount of cellular PTPσ found in the full-length form, there was no suggestion that a similarly large proportion of PTPσ staining was confined to an intracellular compartment. The relative subcellular localisation of the PTPσ species was therefore analysed further biochemically using limited trypsinisation of intact, living cells. Cells expressing PTPσ were incubated in a dilute trypsin solution. This has previously been shown to trim cell surface proteins whilst leaving intracellular proteins unchanged (Olson et al., 1988; Brady-Kalnay and Tonks, 1993). Interestingly, full-length PTPσ was sensitive to digestion with extracellular trypsin whereas cleaved PTPσ was relatively protected (Figure 3.6). Several possible explanations exist for the relative resistance of $E_{90/81}$ to trypsin. Firstly, biochemical differences, perhaps in the post-translational modifications of the different PTPσ species, may protect $E_{81/90}$ but not FL_{166} from trypsin digestion. On the other hand, there may be a tyrpsin-sensitive site close to the plasma membrane. Alternatively, $E_{90/81}$ PTPσ might be protected from trypsin by its inclusion in a larger protease resistant complex. Finally, trypsin cleavage may result in the rapid internalisation of the extracellular domain. However, although these possibilities might mask individual trypsin cleavage sites, it is unlikely that they would have the effect of entirely protecting $E_{81/90}$ from digestion. Therefore, there is likely to be a difference in the subcellular localisation of $E_{81/90}$ and FL_{166}.

There are several variations in the relative subcellular localisation of $E_{81/90}$ and FL_{166} that might result in the picture described above. Firstly, the presence of multiple subcellular populations of each form of PTPσ. Secondly, if trypsinisation causes cell lysis, then mature

membrane localised $E_{81/90}$ but not intracellular full length protein might be protected from trypsinisation by inclusion in "outside-in" micelles. Indeed, in this model the $E_{81/90}$ species might in fact result from trimming of micellar full length protein. However, in these models one might expect a greater reduction both in total protein content and in the $E_{81/90}$ content than that seen. Therefore, it is likely that cleaved PTPσ is indeed cell surface localised and $E_{81/90}$ intracellularly localised. Further work might usefully examine whether the HA-tagged intracellular domain cleavage fragments, $P_{85/75}$, are likewise protected from trypsinisation. As intracellular proteins, their level should not be reduced by trypsin treatment if the cell membrane remains intact. Indeed, their effective level may go up as similar fragments will be produced by trypsinisation of cell surface FL_{166}. Finally, if $E_{81/90}$ is present at the cell surface in the form of E/P subunit heterodimers it may also be possible to detect the loss of the short extracellular domain peptide present in P_{85}, which might function as an indirect measure of the amount of E/P subunit heterodimers located at the cell surface.

In contrast to the data discussed above, previous studies have reported that full-length PTPσ has an intracellular distribution whereas $E_{81/90}$ represents the mature cell-surface form of the receptor (Yu et al., 1992; Streuli et al., 1992; Stoker et al., 1995b). Previous studies have used surface labelling methods, which highlight membrane proteins in contrast to trypsinisation, which selectively digests membrane proteins. However, the two approaches should give comparable results with one notable exception. The labelling methods will irreversibly label all proteins that reach the cell surface during the course of the assay. However, this label will then persist whether or not the protein is cleaved and whether or not it remains on the cell surface. As cleaved PTPσ must necessarily be generated from full length PTPσ, the presence of label on cleaved PTPσ does not necessarily prove that the cleaved form is present on the cell surface. In contrast, proteins marked by trypsinisation do not persist in the same way and the digestion of full length protein means that it must at some stage in its lifetime be localised to the plasma membrane. In addition, the selection of immunoprecipitation or detection antibodies may have contributed to discrepancies between studies. However, it is of interest that no previous study has determined what proportion of each PTPσ species is detected at the cell surface although one study commented that type II RPTP surface labelling is inefficient, implying that only a fraction of these proteins might be present at the cell surface (Gebbink et al., 1995; Krasnoperov et al., 2002). Moreover, although Streuli et al detected full-length LAR after a 15 minute labelling period, the relative level of this species when compared to

the cleaved form meant that it was not detectable in cells labelled for 4-6 hours, which must necessarily contain some protein in this form (Streuli et al., 1992). In systems where little or no full-length protein is seen, only cleaved protein is seen on the cell surface (Streuli et al., 1992; Stoker et al., 1995b). In contrast, in systems where a small proportion of full-length protein is seen, a small proportion of cell surface full-length protein is seen (Yu et al., 1992). The present study has the highest level of full-length protein of these studies and correspondingly has the highest level of cell surface full-length protein. By extension if cleaved protein is located intracellularly, in cells expressing predominantly cleaved protein, most PTPσ must be intracellularly located. This resembles the situation seen with the leech type IIa RPTP, HMLAR2 (Gershon et al., 1998a). Moreover, this thesis represents the first study to compare the total amount of RPTP detected with that located at the cell membrane. The next question that must be answered is what affects the cleavage rate of full-length PTPσ in different expression systems?

The regulation of type IIb RPTP cleavage has been extensively studied. Although less well understood, the amino-terminal cleavage of type IIa RPTPs appears to be regulated in the same way. However, despite the similarities between these two cleavage events, the field does not normally consider them analogous. There is little evidence for this position. Both cleavage events occur at a conserved furin-like cleavage site in the final fibronectin type III repeat (Streuli et al., 1992; Jiang et al., 1993; Serra-Pages et al., 1994; Pulido et al., 1995a; Campan et al., 1996; Fuchs et al., 1996; Cheng et al., 1997; Anders et al., 2006). The expression level of several RPTPs including the type IIa and IIb RPTPs is regulated by cell density and is associated with changes in cellular proliferation rate (Streuli et al., 1992; Ostman et al., 1994; Celler et al., 1995; Gebbink et al., 1995; Campan et al., 1996; Fuchs et al., 1996; Li et al., 1996; Bianchi et al., 1999; Xu et al., 2005). In both protein families, the cleaved form is generally more abundant than the uncleaved form and there is evidence that both full-length and cleaved protein reach the cell surface, although the majority of cellular type IIb RPTP is located intracellularly in low density cells (Gebbink et al., 1995; Phillips-Mason et al., 2006). Strikingly, surface expression of type IIb RPTPs is restricted to focal adhesions where they interact with cadherins and β-catenin (Brady-Kalnay et al., 1995; Gebbink et al., 1995; Fuchs et al., 1996; Brady-Kalnay et al., 1998; Bianchi et al., 1999; Mourton et al., 2001; Schnekenburger et al., 2005; Del Vecchio and Tonks, 2005). These interactions may be important mediators of the effects of cell density on type IIb RPTP level (Taniguchi et al., 1999; Chattopadhyay et al., 2003). The level of type IIa and IIb RPTPs increases in confluent cells due to changes in protein stability,

although it is unclear whether this effect is due to cell-cell contact per se, possibly through cadherin engagement, or due to cell cycle arrest. Type IIa RPTP mRNA also increases in contact-inhibited cells (Celler et al., 1995; Gebbink et al., 1995; Symons et al., 2002). The increase in type II RPTP levels in confluent cells may function to specifically block the induction of proliferation by growth factor signaling in contact inhibited cells (Machide et al., 2006).

The post-translational cleavage of type II RPTPs has also been reported to be affected by cell density. This effect is associated with an increase in the levels of the protease responsible (Campan et al., 1996). In addition, there is a suggestion that the turnover rate is reduced in confluent when compared to sparse cultures. The turnover rate of type II RPTPs is high as would be expected for a protein that behaved in this way (Yu et al., 1992; Streuli et al., 1992). In this thesis it was shown that PTPσ protein levels were stabilised in confluent when compared to sparse cultures (Figure 3.3B). However, the steady state pattern of PTPσ cleavage fragments with FL_{166}, E_{90} and E_{81} was only reached after 64 hours, despite the rapid half-life reported for these proteins (Yu et al., 1992; Streuli et al., 1992). However, previous studies showed that type II RPTP protein levels stabilise over at least 36 hours after induction (Gebbink et al., 1993b; Wang et al., 2000). The increase in PTPσ level may therefore reflect the fact that these proteins appear to stabilise their own expression or be stabilised by increasing cell contacts. Finally, a detailed examination of the data failed to show any clear effect of cell confluency on PTPσ cleavage. However, interpretation of these gels was complicated by a number of factors. The most important being the difficulty encountered loading equivalent amounts of cell lysate into each lane. Unfortunately, the variable contribution of media-derived bovine serum albumin (BSA) to different lysates prevented the direct comparison of equivalent amounts of cell lysate. In addition, it was difficult to determine whether apparent differences in the pattern of PTPσ species seen reflected real differences or artefacts of different relative exposure levels in samples with different amounts of PTPσ. Alternative experimental approaches might minimise the effects of cell proliferation or separate transcriptional and post-translational levels of regulation. This would allow the accurate determination of the effects of cell density on PTPσ expression level and cleavage patterns.

In summary, the experiments presented in this chapter confirmed that PTPσ is expressed initially as a large precursor protein (FL_{166}), which is subsequently cleaved at two sites. Amino-terminal cleavage generates E_{81} and P_{85} fragments. Carboxyl-terminal cleavage generates E_{90} and P_{75} fragments. Sequential amino- and carboxyl-terminal cleavage

generates E_{81}, P_{75} and a juxtamembrane fragment. Neither cleavage event alone is sufficient for shedding of the extracellular domain. It was shown that the majority of cellular PTP is found in the cleaved form and that this is mainly intracellular. In contrast full-length protein is located mainly at the cell surface.

Chapter 4: Disulphide cross-linking of PTPσ oligomers

The regulation of receptor tyrosine kinase activity is well understood whereas very little is known about RPTP regulation. The reasons for this discrepancy are complex but a major factor is that inappropriate or prolonged RTK activation is associated with a range of human and animal conditions whereas RPTP inactivation has in general a much less conspicuous effect. It has therefore been easier to examine the function and regulation of RTKs than RPTPs. Classically, inactive RTKs are monomeric and the binding of specific ligands stimulates dimerisation and receptor activation (Yarden and Schlessinger, 1987; Lemmon and Schlessinger, 1994). However, more recent data suggests that like the insulin receptor and some cytokine receptors, which signal through activation of associated cytoplasmic tyrosine kinases, many RTKs form inactive dimers in the absence of any ligand. In these cases, ligand binding possibly stabilises or stimulates an active dimeric conformation in which each subunit is able to transphosphorylate the other resulting in an active RTK dimer (Schlessinger, 1988; Herr et al., 1997; Livnah et al., 1999; Luo et al., 1999; Moriki et al., 2001; Yu et al., 2002; Li et al., 2005).

RPTPs are similar in many ways to RTKs. Both enzyme families consist of extracellular receptor-like domains and intracellular catalytic domains that catalyse phosphate group transfer at the plasma membrane. For this reason, it is assumed that RPTPs counter-balance RTK activity and it would not be surprising if similar control mechanisms existed to regulate their activity. However, despite these initial similarities there are many areas of difference. In contrast to RTKs, RPTPs isolated from cell membranes are constitutively active (Streuli et al., 1989; Guan et al., 1990). The low phosphotyrosine level seen in unstimulated cells thus may reflect a low unstimulated level of RTK activity and a high basal level of RPTP activity (Hunter and Sefton, 1980). Like some RTKs, RPTPs can exist in dimeric form (Bilwes et al., 1996; Jiang et al., 2000; Tertoolen et al., 2001; Xu and Weiss, 2002). However, it is unclear what proportion is dimeric at the cell membrane. Previous research has revealed RPTP dimers using cross-linking techniques and cellular treatments. In contrast to RTKs, high levels of RPTP dimerisation are associated with RPTP inhibition not activation possibly through steric occlusion of each monomer's active site by a corresponding juxtamembrane wedge domain on the other subunit (Desai et al., 1993; Bilwes et al., 1996; Majeti et al., 1998; Jiang et al., 1999; Xu and Weiss, 2002). However, the physiological role of this mechanism is unclear. This wedge motif is not present in all RPTPs. Furthermore, two catalytic domain RPTPs

are sterically unable to adopt a dimeric conformation opposing the wedge and catalytic domains (Nam et al., 1999; Nam et al., 2005). Therefore, although dimerisation plays a key role in RTK function, the role and even the existence of dimerisation in physiological RPTP function remains unclear.

This chapter describes for the first time, the isolation of a full length RPTP dimer in unstimulated cells. A large-template, site-directed mutagenesis system was developed and used to introduce ectopic cysteine residues into the chick PTPσ cDNA. The introduction of these residues allowed the formation of intramolecular disulphide bonds that cross-linked PTPσ dimers in mammalian cells.

4.1: Introduction of cysteine substitutions by large template site-directed mutagenesis

Disulphide cross-linking is a method that introduces cysteine residues into one or more proteins of interest in order to induce ectopic disulphide bond formation. This allows the scientist to address a variety of structural and biochemical questions. The degree of disulphide bond formation between any two specific cysteine residues provides information on their proximity in the native tertiary or quaternary structure and on the flexibility of the local structure (Falke and Koshland, Jr., 1987; Falke et al., 1988; Lynch and Koshland, Jr., 1991; Pakula and Simon, 1992). In comparison to biochemical crosslinking agents, disulphide crosslinking occurs at specific selected sites only, identifies closer interactions (due to shorter crosslink length) and can occur under physiological conditions, as it usually does not require chemical treatment. Furthermore, cysteine substitution is minimally disruptive to native structure, allowing the examination of sensitive biological responses such as the effects of ligand binding on receptor dimerisation, conformation and activity (Milligan and Koshland, Jr., 1988; Cao et al., 1992; Sorokin et al., 1994; Jiang et al., 1999).

Disulphide cross-linking requires the introduction of several point mutations at a range of sites in the PTPσ cDNA. However, the shorter of the two major PTPσ transcripts is approximately 4.5 kb in length. Constructs containing it are too large for conventional whole-plasmid, site-directed mutagenesis (e.g. Quikchange, Stratagene). Although mutagenesis reactions using a 4kb template produced many mutant PTPσ colonies, use of the 12kb full-length PTPσ expression vector did not produce any colonies. Moreover, in contrast to the reaction employing the shorter template, the full-length template reaction

did not produce detectable amplification product. (Figure 4.1A, lane 3 and 2). Conventional, exponential PCR amplification reactions generated detectable products using templates of this size. Varying the DNA polymerase, the cycle number or the reaction mix components did not improve mutagenesis efficiency using the larger template (Data not shown). This suggested that some factor peculiar to the site-directed mutagenesis reaction was responsible for the failure of these reactions rather than the length of the PCR reaction *per se.*

Alternative mutagenesis strategies were considered. However, all required suitably located, unique restriction enzyme recognition sites in the PTPσ sequence. The length of the PTPσ expression construct means that these are not often available. Therefore, the use of these methods with PTPσ often requires partial digests and several cloning steps. This project required the introduction of a number of different mutations into a variety of PTPσ sites. Using conventional mutagenesis and sub-cloning techniques this would have meant many, possibly technically difficult steps for each mutation. It is likely that a method of this kind would have significantly delayed progress from molecular biology experiments making the mutant constructs to *in vivo* experiments testing the effect of the mutations. Therefore, a large-template, whole-plasmid mutagenesis system was developed and used to introduce the cysteine substitutions.

4.1.1: Development of a large template site-directed mutagenesis method

Most of the important variables in whole plasmid amplification are the same as those in conventional polymerase chain reactions. However, there are specific features of site-directed mutagenesis reactions that make them less efficient than conventional polymerase chain reactions.

Firstly, the complementary mutagenic primers used in site-directed mutagenesis reactions make primer-dimer formation more favourable than primer-template binding. The likelihood of productive primer-template binding can be favoured in several ways. The use of long primers minimises the relative effect of the mutation on primer-template binding affinity. However, excessively long primers again favour primer-dimer formation (Wang and Malcolm, 1999). The template level in site directed mutagenesis reactions is also higher than would be expected in a conventional PCR reaction to maximise the likelihood of primer-template collisions. In addition, primer template binding is improved by carrying out reactions with only a single primer, or using two initial half-reactions utilising only one

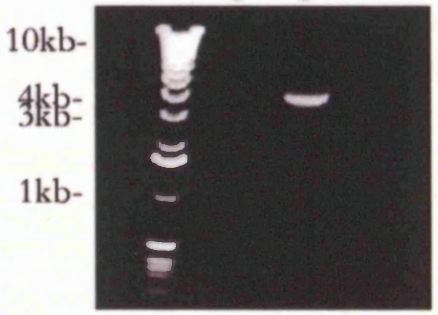
primer each, which are later combined to make a whole 2-primer reaction for the remaining cycles (Wang and Malcolm, 1999).

Secondly, mutagenic primers bind more strongly to mutated product than to template. However, mutagenic primer can only bind to the extreme 3' end of the mutated product (Figure 4.1D). Therefore such primer-product complexes cannot be extended by DNA polymerases. Therefore, site directed mutagenesis reactions exhibit linear rather than exponential amplification and this results in a lower yield. In addition, as mutated product accumulates it antagonises further productive cycles and so increasing cycle number is not useful in these reactions.

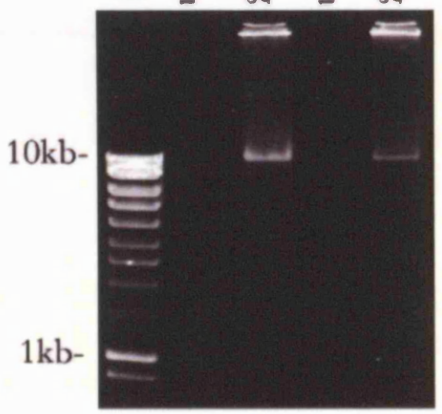
A range of different primer and template concentrations were tried together with a number of cycle parameters (Data not shown). However, it did not prove possible to achieve successful conditions for large template site directed mutagenesis using any of these conditions.

Increasing the length of oligonucleotide primers does not improve the efficiency of large template mutagenesis (Wang and Malcolm, 1999). However, splice-overlap extension PCR commonly uses 2 pairs of perfectly complimentary megaprimers in excess of 100bp and 1 pair of flanking oligonucleotide primers to generate products. It may be that the exponential nature of these reactions allows them to succeed where site directed mutagenesis does not. However, it may be that above a certain length, which it is not practical to reach using synthetic oligonucleotides, large primers begin again to work. Such megaprimers would be long enough that the decrease in binding affinity due to the mutation would be almost undetectable. The optimum size for megaprimers in such reactions is about 300bp (Barik and Galinski, 1991; Lai et al., 2003). The Quikchange method was easily adapted to use 300bp megaprimers generated by a first stage reaction instead of the synthetic mutagenic oligonucleotides conventionally used.

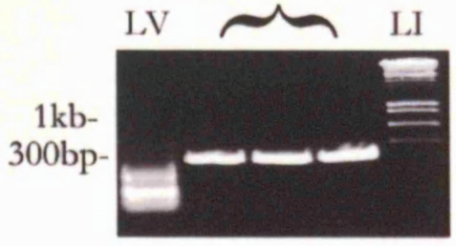
A


L+
pCMV25 FL - 12kb
pBS TM - 4kb
10kb-
4kb-
3kb-
1kb-

B


LI
Pre-Pfu
Post-Cycling
Pre-Pfu
Post-Cycling
10kb-
1kb-

C


LV
1st stage PCR product
LI
1kb-
300bp-

D

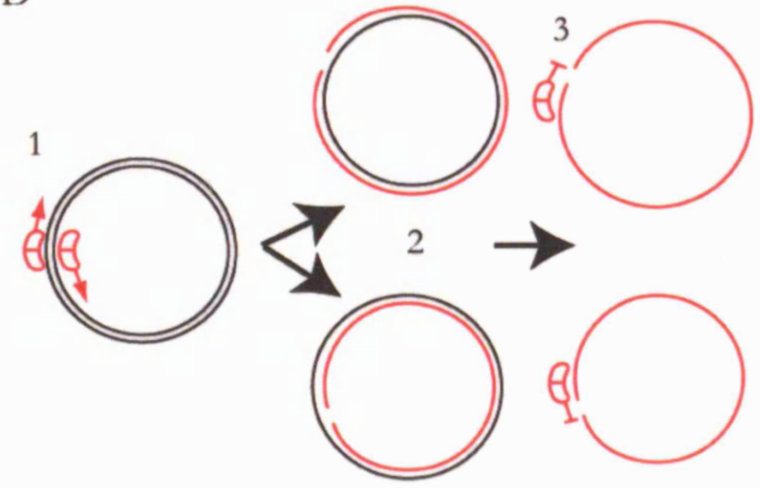

1
2
3

Figure 4.1: Development of large-template site-directed mutagenesis method

A. Failure of standard mutagenesis system using large templates: Agarose gel electrophoretic analysis of the products of standard Quikchange (Stratagene) mutagenesis reaction. The same primers were used with equimolar amounts of either a 12kb template (Lane 2) or a 4kb template (Lane 3) with appropriately adjusted reaction profiles. L+=Gibco 1kb+ Marker (Lane 1). Marker bands are as indicated.
B. Efficient production of 12kb mutagenesis product using a modified two-stage protocol. Equal volumes of PCR reaction pre-enzyme addition (Lanes 2, 4) and after thermal cycling (Lanes 3, 5). LI=Bioline Hyperladder I (Lane 1). Marker bands are as indicated.
C. Production of megaprimer for two stage modified mutagenesis protocol: Agarose gel electrophoresis of the short PCR product (Megaprimer – Lanes 2,3,4) produced in the first stage of the modified large template mutagenesis system. LV=Bioline Hyperladder V (Lane 1), LI=Hyperladder I (Lane 5) Marker bands are as indicated.
D. Schematic depiction of whole plasmid PCR method. 1: Complimentary mutagenic primers anneal to the (methylated) template of interest. 2: Primer extension generates complimentary unmethylated single-stranded, linear DNA containing the desired mutation. 3: In subsequent cycles, primer is a better match for product than template but is unable to extend the new product as the sequence is broken in a 5'→3' direction immediately after the site recognised by the primer.

Large template mutagenesis using PCR generated mutagenic megaprimers successfully generated 12kb mutated plasmids. Replacing one of the complementary primers with a primer 300bp downstream produced abundant first stage PCR product (Figure 4.1C). The entire megaprimer product was used as a primer in a second stage reaction following electrophoresis and gel extraction to remove unwanted DNA species (Smith and Klugman, 1997). The second PCR reaction produced a high molecular weight band that migrated at the same size as linearised plasmid DNA (Figure 4.1B) and the appearance of this linearised plasmid DNA was often associated with a decrease in the amount of megaprimer seen following the reaction (Data not shown). The second stage PCR product was then digested with *DpnI* and transformation of a fraction of this product recovered many clones of mutated plasmid DNA. Mutant clones were verified by digestion (if applicable) followed by DNA sequencing of the PTPσ coding region and test expression studies. No undesired mutations were detected in any of the mutants containing the mutation of interest.

The method described above is similar to one recently published for transferring mutations between plasmids (West and Wilson, 2002). The only major difference between the method described here and that published by West and Wilson is that the method described here uses larger template sizes and employs the PCR additive, Betaine. Experiments using this method generated more than 20 independent mutations in plasmids ranging from 4 to 15kb in size and also allowed the transfer of mutations between constructs. Sequence analysis has revealed no additional amplification errors in the coding regions of these constructs. However, despite optimisation some primers do not generate second-stage, mutated products although they efficiently generate first-stage products. Moving the primer binding sites by as little as a couple of nucleotides in either direction overcomes this problem (Michael West, personal communication and data not shown). This method is a robust protocol that can be easily used to generate mutated plasmids of over 12kb in size.

4.1.2: Selection of sites for cysteine substitution

The selection of suitable sites for cysteine substitution is an essential part of any disulphide cross-linking project. Guidelines have previously been established for the selection of suitable sites and these will be addressed in detail below with particular reference to PTPσ (Falke and Koshland, Jr., 1987). Briefly, mutations at such sites need to

be unlikely to interact with endogenous cysteines, surface exposed and non-disruptive to receptor function.

Firstly, to ensure that substituted cysteines do not interact with endogenous unpaired cysteines it is essential to identify the sites of the endogenous cysteine residues. Chick PTPσ (short form with four Fibronectin type III domains) contains twenty cysteines distributed throughout the protein, rather than clustered in a cysteine rich region as seen in some other extracellular receptors. Seven cysteine residues are located extracellularly, one in the transmembrane domain and twelve intracellularly. The predicted signal peptide includes one cysteine residue, which is therefore probably not found in the mature protein. Each of the three immunoglobulin domains contains a disulphide bond, which together account for the remaining six extracellular cysteine residues. Therefore, all the cysteine residues that are present in the extracellular domain of mature PTPσ protein are already involved in disulphide bonds and hence are unlikely to interact with ectopic cysteine substitutions. The remaining twelve cysteines are intracellular. One is located just inside the membrane in a position likely to be involved in palmitoylation, which may play a role in the localisation of RPTPs to lipid rafts (Pace et al., 1999; Yik and Weigel, 2002; Caselli et al., 2002; Bijlmakers and Marsh, 2003). Two more are located close together between the two phosphatase domains and there are five in the first and four in the second phosphatase domain including the two catalytic site cysteines. However, in mammalian systems the reducing intracellular environment and potent intracellular and membrane-associated disulphide reductases reduce the likelihood of the formation of stable disulphide bonds in these regions. Therefore, it is not appropriate to locate the cysteine substitution sites in the intracellular region and the extracellular, juxtamembrane and transmembrane domains were analysed further for suitable substitution sites.

Secondly, primary sequence comparison of PTPσ with closely related proteins identified conserved sequences likely to be important for receptor function. Overall, the primary sequences of PTPσ orthologues from different species showed a very high degree of conservation (Data not shown). This was particularly marked in the transmembrane and catalytic domains with the extracellular domain and juxtamembrane regions being less well conserved when PTPσ was compared with orthologues and particularly paralogues from other species. This suggests that the selective pressure against mutations is less in the extracellular and juxtamembrane regions than the transmembrane region and so these regions are less critical for the function of type II RPTPs.

Thirdly, residues need to be exposed on the protein surface if they are to be able to interact and form disulphide bonds. The structure of the non-catalytic regions of type II RPTPs has not yet been determined and so surface exposure can only be predicted based on amino acid composition and fold. Transmembrane domains form α-helices such that all the amino acid side-groups project outwards. However, PTPσ has an endogenous transmembrane cysteine residue that is notably conserved in all known PTPσ and PTPδ proteins but absent in all known LAR proteins (Figure 4.2A). This pattern of conservation might indicate an association with the different characteristics of PTPσ/δ and LAR, which would complicate the functional interpretation of the effects of any cysteine substitutions in this region. In addition, it might be difficult to distinguish between disulphides comprised of one endogenous and one ectopic cysteine and those comprised of two ectopic cysteines. Furthermore, the interactions between dimeric receptors are complex but often involve transmembrane domain interactions (Weiner et al., 1989; Moriki et al., 2001; Constantinescu et al., 2001; Fleishman et al., 2002; Mendrola et al., 2002; Chin et al., 2005; Choi et al., 2005; Kjaer et al., 2006). Therefore, because of its possible role in dimer interactions the transmembrane domain is not an ideal target for cysteine substitution. The juxtamembrane region often continues the alpha-helical structure of the transmembrane region. Therefore, the juxtamembrane region was subjected to further analysis to determine suitable sites for cysteine substitution.

Lastly, the transmembrane domain has several features that might affect the location of cysteine substitutions sites. Hydrophobicity analysis of the juxtamembrane region shows that the most charged region is centred on residue 838 and that a second highly charged region is located from residues 846-9(Figure 4.2B). These two regions are therefore more likely to be surface exposed than buried and so might provide suitable target regions for cysteine substitution. However, the highly hydrophilic group of residues (846-9) just outside the predicted transmembrane domain is likely to determine the orientation of membrane insertion together with the corresponding positively charged cluster on the opposite side of the membrane (von Heijne, 1989; Beltzer et al., 1991). It is likely that disruption of this region might have significant effects on protein structure. This region also contains a proline rich region (839-43). Proline has particular characteristics that often play an important role in protein structure, breaking α-helices. However, as there are several prolines the loss of any one proline should be well tolerated. Moreover, the proline-rich region is only poorly conserved: RPTPδ lacks one proline, LAR lacks two and their presence is variable in type IIb RPTPs. Therefore, the cysteine substitution sites were

located in the proline-rich region (839-843) in proximity to the polar region centred on residue 838.

Dimerisation *per* se is not sufficient for RTK activation and has specific effects depending on the orientation of the constituent monomers to each other (Burke and Stern, 1998; Jiang et al., 1999). Therefore, disulphide crosslinking might have different effects on PTPσ function depending on the orientation imposed on the dimers by each disulphide bond. As transmembrane domains and their immediate juxtamembrane surrounds are considered α-helical in nature, mutations at every other residue over a range of eight to ten amino acids will place a potential disulphide bond site on most of the potential interaction faces. If the protein in question dimerises then this ensures that at least some of the substitution sites are in an appropriate orientation for disulphide bond formation. Furthermore, if multiple cross-links form then the properties of the dimers may vary according to the relative orientation of the constituent monomers. Therefore, five cysteine substitutions were made at alternate sites in the juxtamembrane region (D837C, P839C, P841C, P843C, I845C – Figure 4.2C).

4.2: Expression of cysteine-substituted PTPσ

Although the cysteine substitutions were located in such a way as to reduce the likelihood that they would have significant effects on PTPσ structure, it is important to identify whether they do disrupt any of the known characteristics of PTPσ such as its sub-cellular localisation and post-translational processing. As discussed above, PTPσ undergoes post-translational cleavage at two sites resulting in five distinct protein species. (Chapter 3) These correspond to mature FL protein (Fl_{166}), the products of amino-terminal cleavage (E_{90}, P_{75}) and the products of carboxy-terminal cleavage (E_{81} P_{85}). It is impossible to determine with the current materials whether either of the small fragments (P_{75}, E_{81}) is the product of a single cleavage event or the product of consecutive amino- and carboxy-terminal cleavage. To allow the products of cleavage to be followed, tags were incorporated in the recombinant PTPσ proteins at both the N- (FLAG) and C-termini (HA/Myc). Both tags detect full-length protein (FL_{166}).

A.

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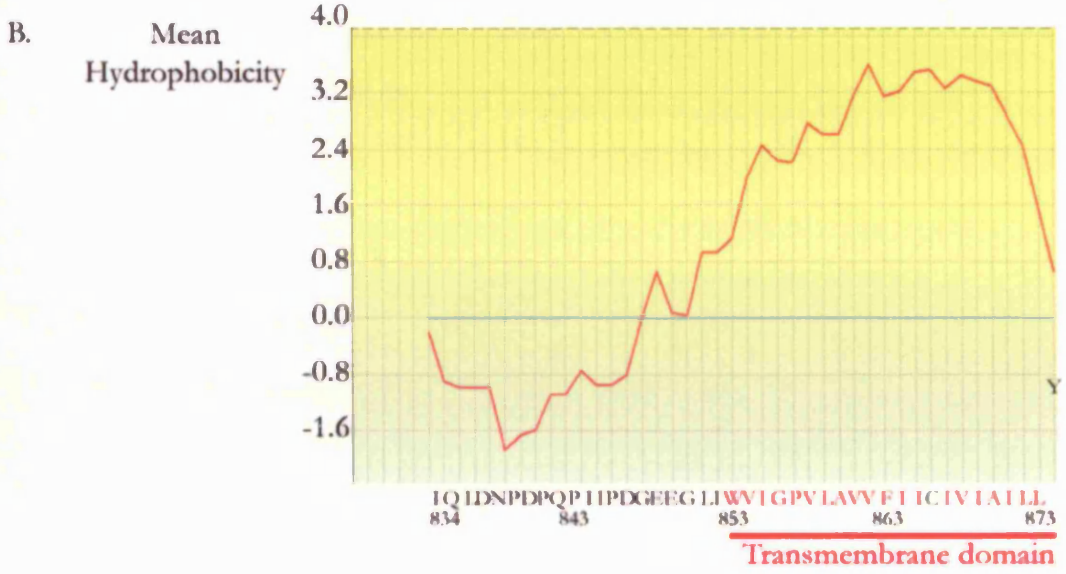
Chick RPTPσ: FSDPIQLDNPDPQPIIDGEEGLIWVIGPVLAVVFIICIVIAILLYKNK
Mouse RPTPσ: FSDPFQLDNPDPQPIVDGEEGLIWVIGPVLAVVFIICIVIAILLYKNK
Mouse RPTPδ: YSDPVVSMDLDPQPITDEEEGLIWVVGPVLAVVFIICIVIAILLYKRK
Mouse RPTPf: SPYSDEIVVQVTPAQQQEEPEMLWVTGPVLAVILIILIVIAILLFKRK
                                                 ↑ TM cysteine

Mouse RPTPκ: KAAATEEPEVIPDPAKQTDRVVKIAGISAGILVFILLLLVVIVIVKKS
Mouse RPTPρ: TKGASTQNSNTVEPEKQVDNTVKMAGVIAGLLMFIIILLGVMLTIKRR
Mouse RPTPμ: ATKGAVTPKPVPEPEKQTDHTVKIAGVIAGILLFVIIFLGVVLVMKKR
Mouse RPTPλ: KAACKESKRPLEVSQRSEEMGLILGICAGGLAVLILLLGAIIVIIRKG
```

B.



C.

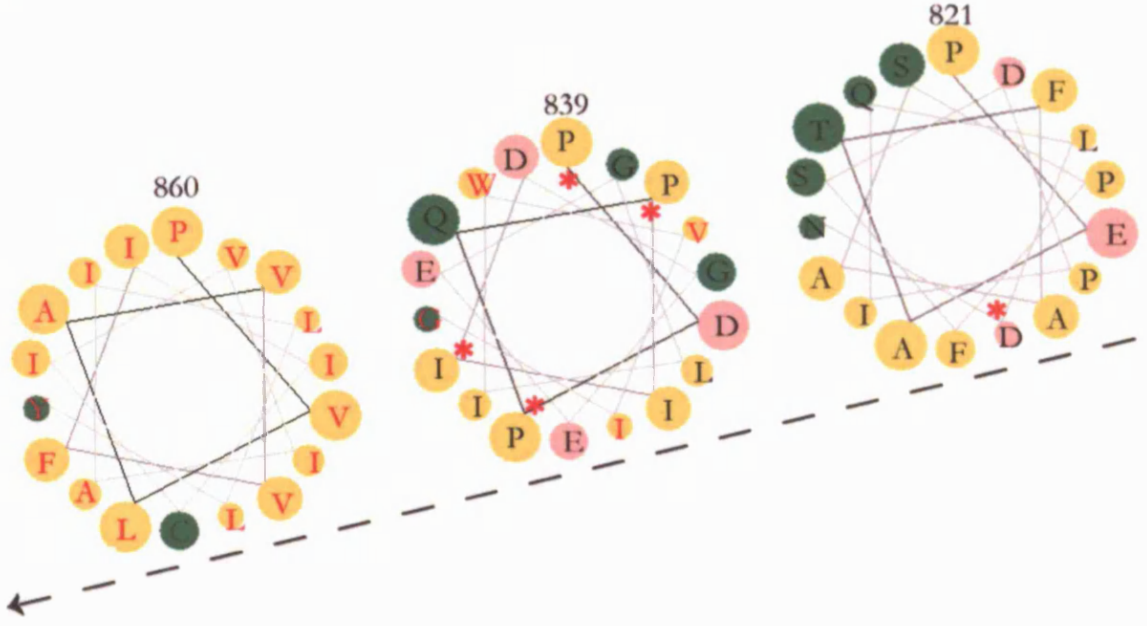
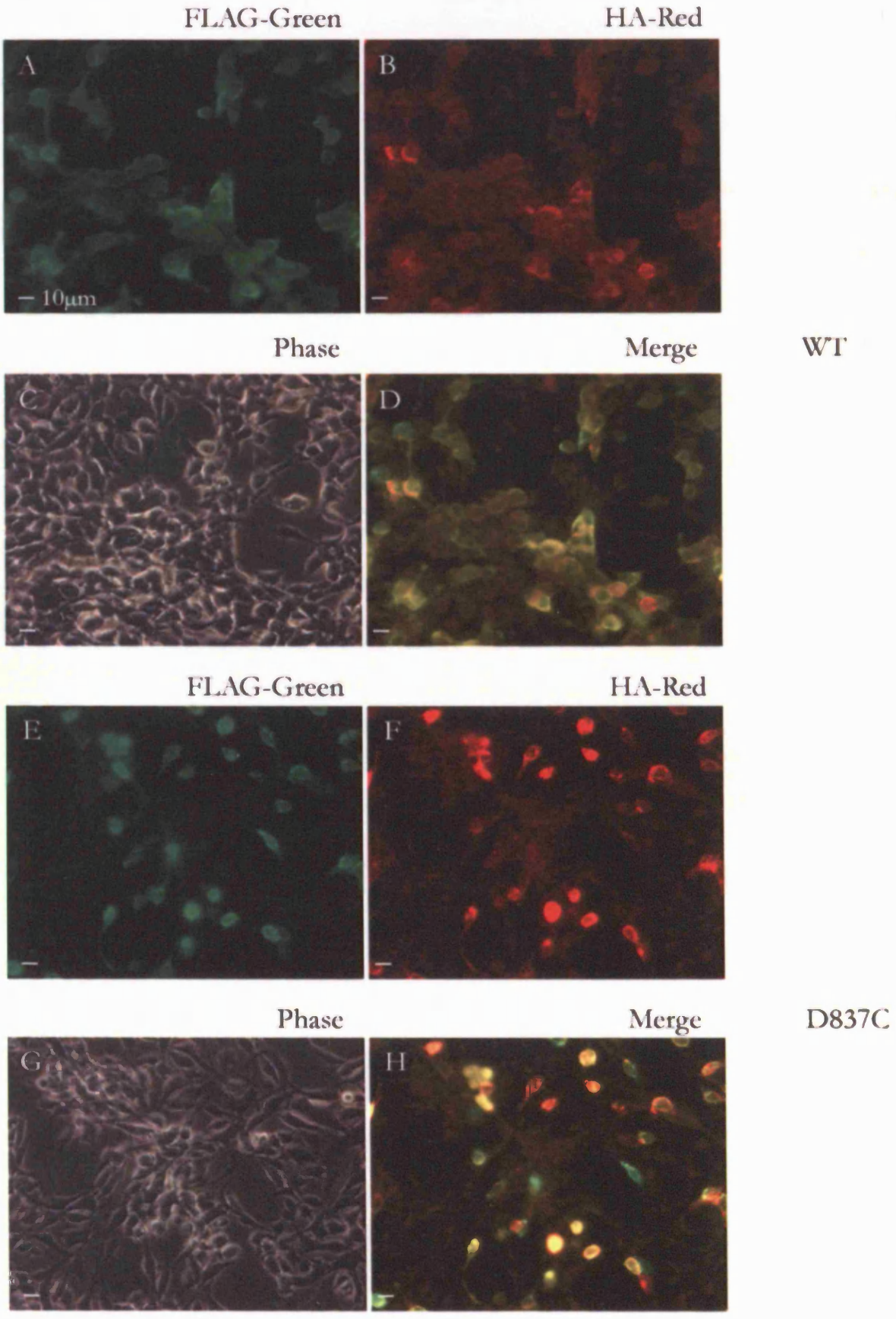


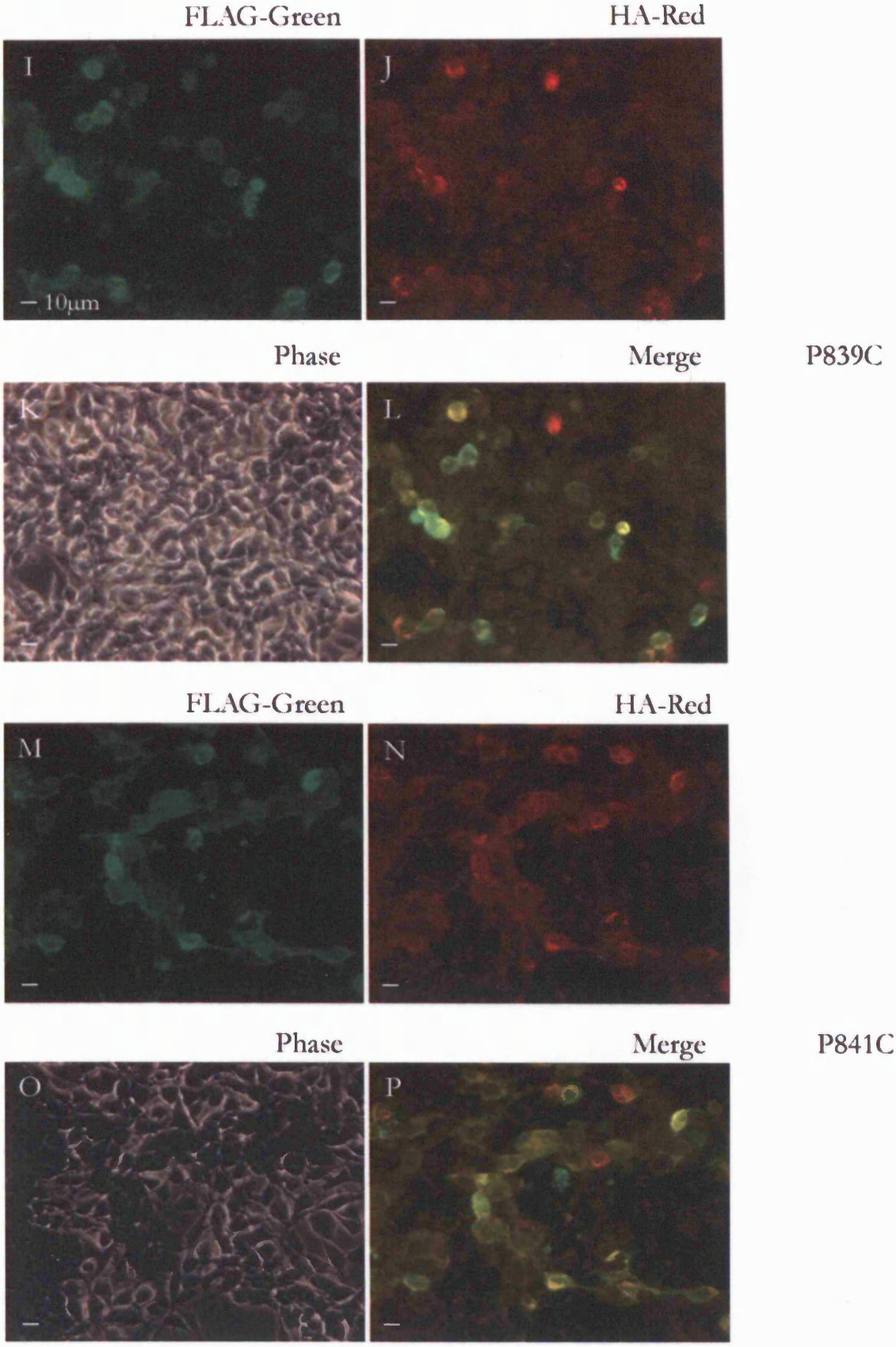
Figure 4.2: Analysis of PTPσ juxtamembrane region

A. Multiple alignment of chicken PTPσ and mouse type II RPTPs (Type IIa – Top; Type IIb – Bottom). Predicted transmembrane helices are highlighted in red (Sonnhammer et al., 1998; Krogh et al., 2001). The site of the transmembrane cysteine that is conserved in PTPσ and PTPδ is marked in dark green and indicated with an arrow. The leucine present at this site in LAR is indicated in pale green.
B. Kyte-Doolittle mean hydrophobicity profile of PTPσ juxtamembrane region. The transmembrane domain is underlined in red. A window size of 9 amino acids was used as this is optimal for evaluating surface exposure (Kyte and Doolittle, 1982). The predicted transmembrane helix is highlighted in red. The site of the transmembrane cysteine that is conserved in PTPσ and PTPδ is marked in dark green.
C. Helical diagrams of PTPσ juxtamembrane region. The helices start at the top-centre of the right hand diagram. The path of each helix is marked by the central spiral and the three diagrams are arranged in the same orientation as each other. Red asterisks indicate sites of cysteine substitution. Yellow residues are non-polar, green residues are polar but non-charged and pink residues are polar and charged. The residues of the predicted transmembrane helix are printed in red characters, and the site of the transmembrane cysteine that is conserved in PTPσ and PTPδ is printed using a dark green characters.

4.2.1: Immunofluorescent analysis of cysteine-substituted PTPσ proteins

Transfection of HEK-293T cells with plasmids containing each of the cysteine substitutions resulted in their expression. The immunofluorescent localisation of wild-type PTPσ is included again for the purpose of comparison. However, as this has previously been discussed in detail, a full description will not be repeated here (3.2.1). No significant difference could be detected in the pattern of cysteine substituted PTPσ expression when compared to wild-type PTPσ. Nor was there any obvious difference in the morphology or behaviour of cells transfected with any of the PTPσ constructs (Figure 4.3).


FLAG-Green
HA-Red
A
B
10μm
Phase
Merge
WT
C
D
FLAG-Green
HA-Red
E
F
Phase
Merge
D837C
G
H


FLAG-Green
HA-Red
I
J
– 10μm
Phase
Merge
P839C
K
L
FLAG-Green
HA-Red
M
N
Phase
Merge
P841C
O
P

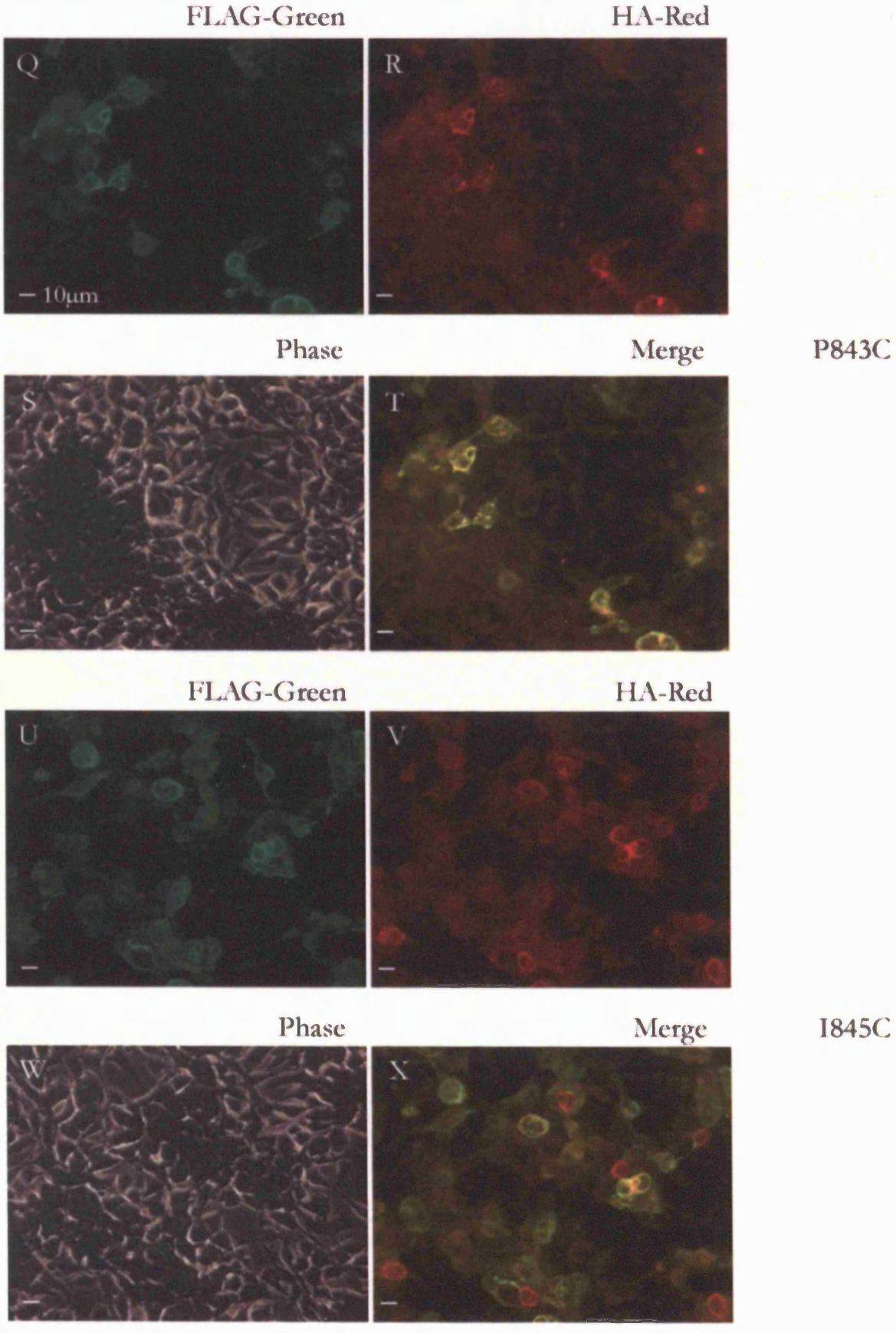

FLAG-Green
HA-Red
Q
R
10μm
Phase
Merge
P843C
S
T
FLAG-Green
HA-Red
U
V
Phase
Merge
I845C
W
X

Figure 4.3:Immunofluorescent analysis of cells transfected with N- and C-terminal tagged PTPσ

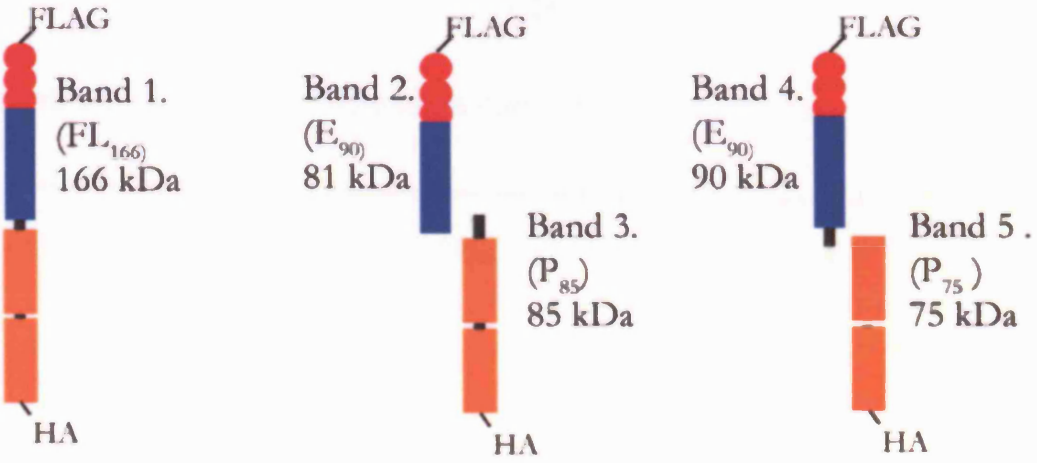
A-D – WT; E-H – D837C; I-L – P839C; M-P – P841C; Q-T – P843C; U-X – I845C . HEK 293T cells were grown on polyethyleneimine-coated coverslips and transfected using the calcium phosphate method with vectors encoding the cysteine-substitution PTPσ sequences. These constructs carry an N-terminal FLAG tag, detected with FITC-labelled secondary antibody (green) and a C-terminal HA tag, detected with Cy3-labelled secondary antibody (red). Scale bars (10μm) are included in each image. All images were obtained at 400x magnification using Axiovision software on an Axioskop II microscope (Zeiss).

4.2.2: 1D SDS-PAGE analysis of cysteine substituted proteins under reducing conditions

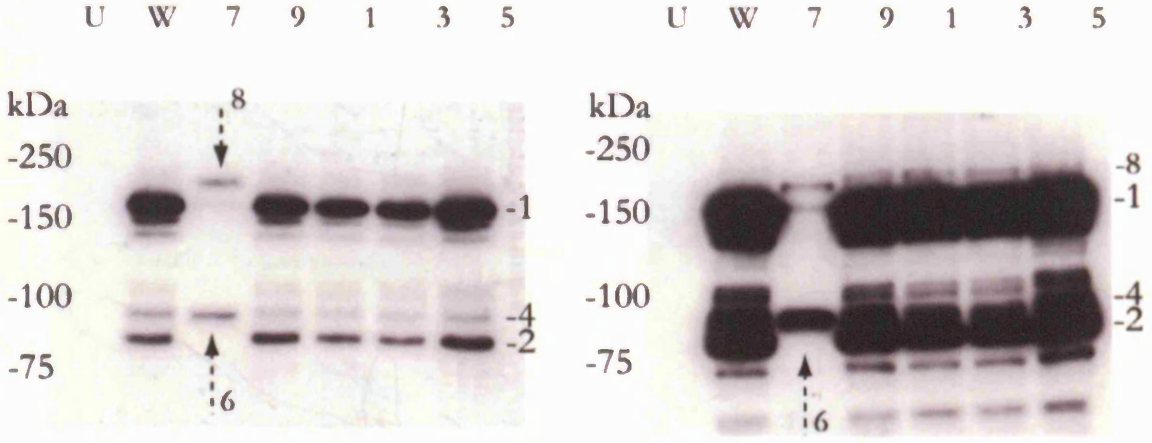
Although sub-cellular localisation of PTPσ did not appear to be affected by cysteine substitution, it is possible that post-translational processing is. Detergent lysis of transfected cells and subsequent SDS-PAGE analysis of these lysates under conventional, reducing conditions revealed a range of PTPσ peptides. As described above, wild-type PTPσ expression results in the presence of five peptide fragments corresponding to the full-length protein (FL_{166}) and four distinct cleavage products (E_{90}, P_{75}, E_{81} P_{85}). The nature and variability of these five PTPσ species was discussed above and will not be further discussed here (3.1).

On reducing SDS-PAGE analysis, the P839C, P841C, P843C and I845C mutants were indistinguishable from wild type protein both in the pattern of cleavage fragments and in their relative abundance. Detection with the FLAG (Amino-terminal) tag revealed three major bands (FL_{166}, E_{90} and E_{81}) of which FL_{166} was the most abundant followed by E_{81} and then E_{90} (Figure 4.4B – bands 1, 2 and 4). Analysis with the HA (Carboxy-terminal tag) again shows three major protein bands (FL_{166}, P_{85} and P_{75}) with a similar pattern of relative abundance (Figure 4.4C). No significant difference was observed in the pattern or level of cleavage fragments obtained with wild type, P839C, P841C, P843C and I845C PTPσ.

A. Schematic diagram of PTPσ cleavage fragments



B. FLAG Detection (Short exposure) (Long exposure)



C. HA Detection (Short exposure) (Long exposure)

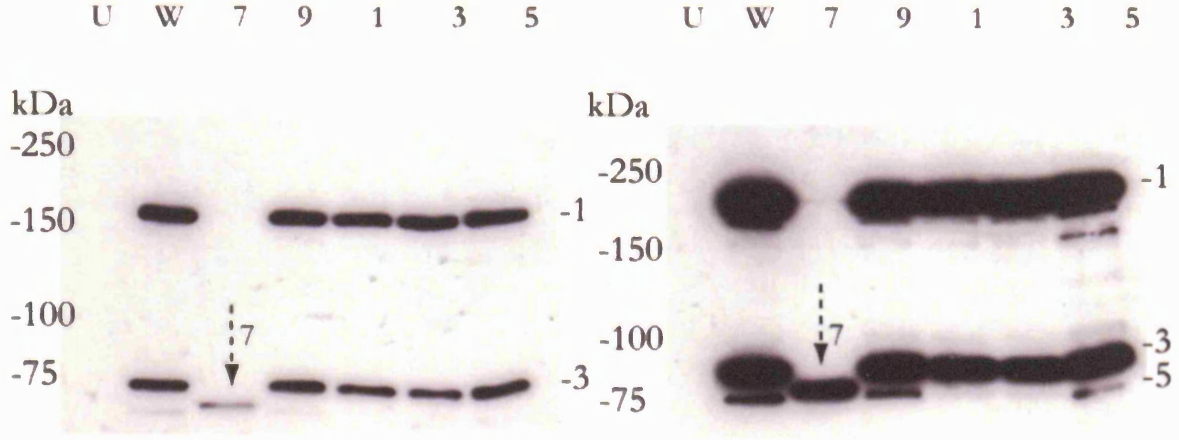


Figure 4.4: Comparative SDS-PAGE analysis of wild-type and cysteine-substituted PTPσ under reducing conditions.

A. Schematic diagram of PTPσ proteins to show tag locations and expected cleavage products.

Protein lysates (**U**ntransfected, **w**ild type, D83**7**C, P83**9**C, P84**1**C, P84**3**C, P84**5**C) were analysed by reducing SDS-PAGE. Figure 4.4 and Figure 4.8 were run in parallel analysing the same samples under reducing and non-reducing conditions respectively.

Proteins transferred to a membrane were probed with antibodies against the amino-terminal FLAG (B) or carboxy-terminal HA tags (C). Longer exposures show the pattern of bands seen with the D837C mutant.

Cleavage of D837C mutant protein produces differently sized extracellular (Band 6 – E_{837}) and intracellular (Band 7 – P_{837}) fragments from other PTPσ proteins.

A band (8) is visible at approximately 170kDa in all the cysteine mutant lysates but not in the wild type lysates. This band (8) is not seen in HA detections of these samples.

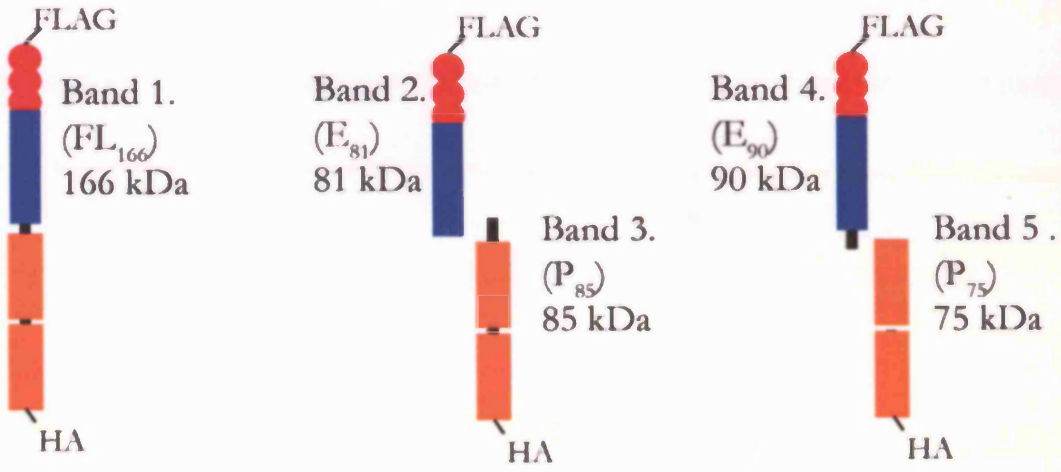
1. Full-length PTPσ 1(FL_{166})
2. Extracellular domain from amino terminal cleavage (E_{81})
3. Phosphatase domain from amino-terminal cleavage (P_{85})
4. Extracellular domain from carboxy-terminal cleavage (E_{90})
5. Phosphatase domain from carboxy-terminal cleavage (P_{75})
6. Extracellular domain fragment from D837C mutant (E_{837})
7. Phosphatase domain fragment from D837C mutant (P_{837})
8. FLAG positive, HA negative, Mw~170kDa.

Under the same conditions, the D837C mutant produced a different pattern of bands. This distinction was also observed in other cell lines transfected with this construct (Data not shown). Firstly, the level of this protein was always lower than wild-type or any other mutant (Figure 4.4B-C). Secondly, the decrease in the total PTPσ level was associated with a decrease in the relative amount of full-length compared to cleaved protein. In addition, the cleavage products of the D837C mutant were qualitatively different from those of other mutants; in most gels only a single FLAG-labelled and a single HA-labelled band were detected. Furthermore, the size of these bands were subtly different from the $E_{90/81}$ and $P_{85/75}$ bands seen with wild type PTPσ or the other mutants and so will be referred to as E_{837} and P_{837} to distinguish them from the conventional cleaved PTPσ forms. Lastly, it was only very rarely possible to detect more than a single band in the region of the cleaved PTPσ fragments and this was usually in the context of a gel where numerous other bands possibly representing, degradation or partial translation products or non-specific detection by the FLAG antibody were detected in this region. (Figure 4.6 B,C)

An additional band (Figure 4.4 – band 8) was detected on longer exposures of the amino-terminal detection of cysteine mutant 839C-845C samples. In contrast, this band was clear on short exposures of the D837C mutant, possibly because the reduced amounts of the full-length form, which is similar in size, do not interfere so much in this mutant. This band was not detected in wild type samples nor on HA detections. The nature of this band will be discussed further when the pattern of bands seen on non-reducing SDS-PAGE is described (4.3).

Type IIa RPTPs such as PTPσ are cleaved and shed by cells that express them (Streuli et al., 1992; Pulido et al., 1995a; Aicher et al., 1997). The function of this secreted extracellular domain is unknown but it may act as a ligand or by binding unknown PTPσ ligands antagonising their interaction with membrane-bound PTPσ. It was not possible to detect shed extracellular domain in the D837C mutant whereas the remaining mutants did not generate reproducibly different levels of the shed extracellular domain than the wild type protein (Figure 4.5). Furthermore, it was not possible to detect disulphide crosslinked forms of the shed ectodomains, suggesting that as expected, these species do not contain the ectopic cysteine residues.

A. Schematic diagram of PTPσ cleavage fragments



B. FLAG detection of conditioned media

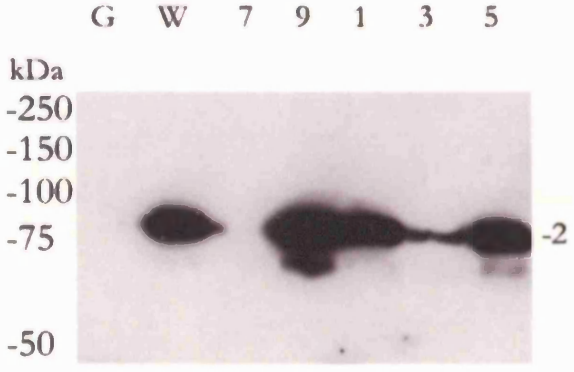


Figure 4.5: Effect of cysteine substitutions on the amount of soluble extracellular domain fragment present in the media of transfected cells.

A. Schematic diagram of PTPσ proteins to show tag locations and expected cleavage products.

Conditioned media (**G**FP transfected, **w**ild type, D83**7**C, P83**9**C, P84**1**C, P84**3**C, P84**5**C) from HEK 293T cells was analysed by non-reducing SDS-PAGE. Proteins were transferred to a membrane and probed with an antibody directed against the amino-terminal FLAG tag.

Band 2. Shed PTPσ extracellular domain (E_s)

4.3: Detection and analysis of disulphide cross-linked PTPσ proteins

Comparison of the cysteine mutants with wild-type PTPσ revealed only limited detectable differences in the processing of the D837C mutant but no difference in its localisation when compared to wild type PTPσ. On the other hand, no differences at all were detected between wild type and the remaining (839-45C) mutants. Following the introduction of an unpaired juxtamembrane cysteine, dimeric PTPσ should form disulphide cross-linked dimers if the cysteines are brought close together in the dimeric structure. Disulphide cross-linked dimers of this kind are readily detectable using non-reducing SDS-PAGE.

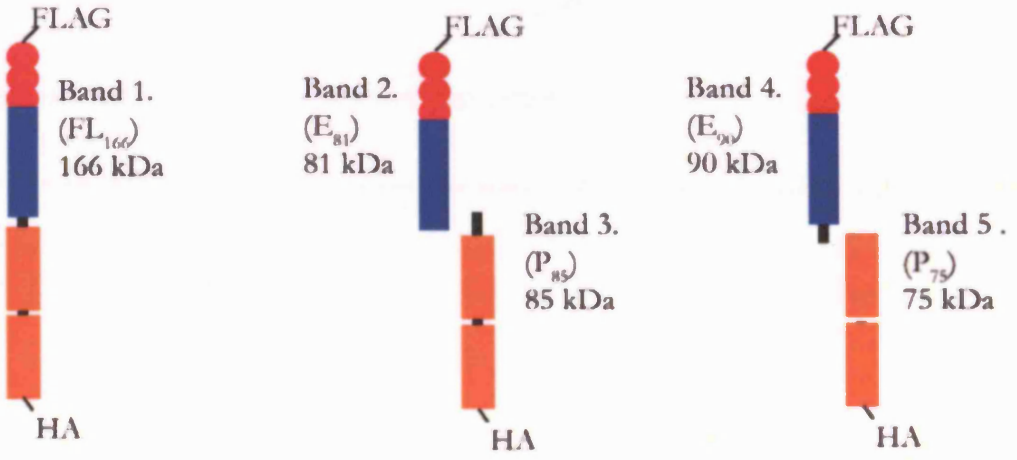
4.3.1: Non-reducing one-dimensional SDS-PAGE without cysteinyl-modifying reagents

Analysis of the wild type and cysteine substituted PTPσ proteins under non-reducing conditions using the amino-terminal FLAG tag showed that there was no difference between the migration of wild type and cysteine substituted PTPσ proteins (Figure 4.6). There was a difference in the apparent size of PTPσ species under reducing and non-reducing conditions. This is presumably due to the different shape of the species as they interact with the pores of the gel. In summary, non-reduced PTPσ species run faster than reduced proteins. To varying degrees, all of the PTPσ proteins ran as four main forms. E_{81}, E_{90} and FL_{166} have previously been described but a significant amount of protein was detected as a previously unseen form, a high molecular weight smear (†) that migrated at considerably greater than 200 kilodaltons. This band was not present under reducing conditions and furthermore the disappearance of the high molecular weight smear under reducing conditions coincided with an increase in the relative amount of full-length protein relative to cleaved extracellular fragment. A similar picture was seen with the chemical cross-linker BS_3 and no clear dimeric bands were detectable in the region of high molecular weight smearing. This suggested that the full-length form of both wild type and cysteine substituted PTPσ proteins might be forming high molecular weight species compatible with disulphide cross-linked dimer formation.

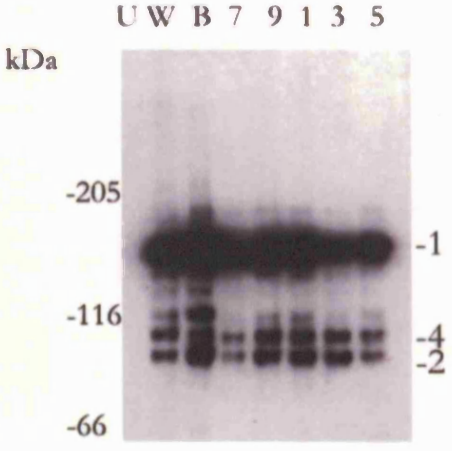
There are a limited number of cysteines in the wild type PTPσ sequence that could mediate wild type PTPσ disulphide bond formation. Intracellular cysteines are unlikely to mediate disulphide bond formation due to the relatively reducing nature of the intracellular environment. Furthermore, work on PTPα demonstrated the lability of disulphide bonds in lysates, presumably to the release of intracellular disulphide bond reductases. There are no

free extracellular cysteines available for disulphide bond formation, all are involved in intramolecular disulphide bond formation within the immunoglobulin domains. The most likely candidate is a transmembrane cysteine residue. Transmembrane cysteines are not uncommon and are able to form disulphide bonds (Rutledge et al., 1992; Moriki et al., 2001; Lu et al., 2006). It is striking that the PTPσ transmembrane domain cysteine is absolutely conserved in PTPσ and PTPδ in all species in which it has been characterised. However, this cysteine is also strikingly absent from LAR in all species characterised and LAR has not been reported to form reduction-sensitive high molecular weight species (Streuli et al., 1992). Mutation of the PTPσ transmembrane cysteine either to a LAR-like sequence (C866L mutation), or to the structurally similar serine (C866S) mutation did not have a significant effect on the formation of a high molecular weight smear when PTPσ peptides were analysed by non-reducing SDS-PAGE (Figure 4.6 D., E.).

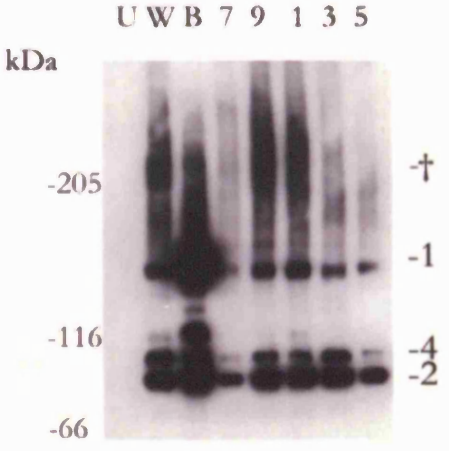
A. Schematic diagram of PTPσ cleavage fragments



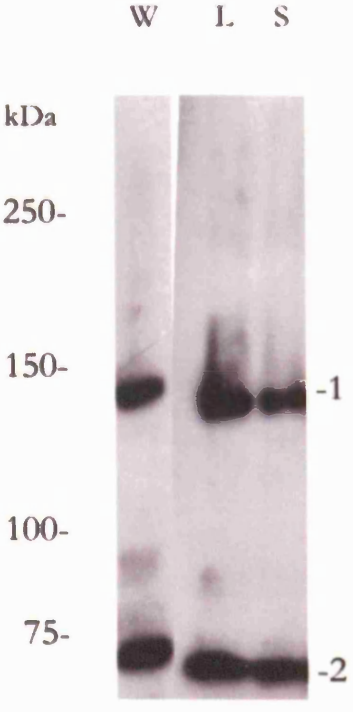
B. Reduced SDS-PAGE
FLAG detection



C. Non-reduced SDS-PAGE
FLAG detection



D. Reduced SDS-PAGE
FLAG detection



E. Non-Reduced SDS-PAGE
FLAG detection

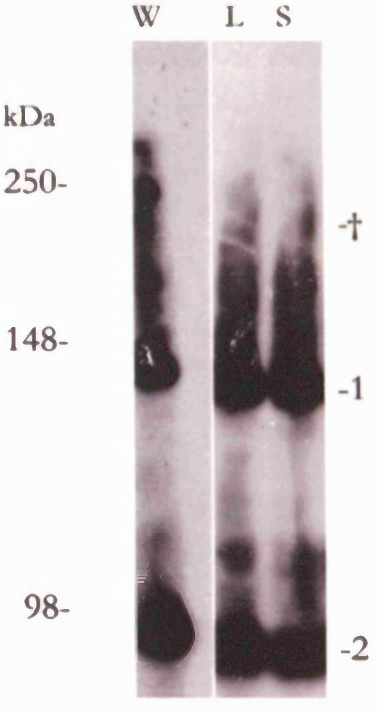


Figure 4.6 : Comparative SDS-PAGE analysis of wild-type and cysteine-substituted PTPσ under reducing and non-reducing conditions in the absence of iodoacetamide

A. Schematic diagram of PTPσ proteins to show tag locations and expected cleavage products.

B., C. Protein lysates (**U**ntransfected, **w**ild type, BS_3 D83**7**C, P83**9**C, P84**1**C, P84**3**C, P84**5**C) were analysed by reducing (B) and non-reducing (C) SDS-PAGE. BS_3= Wild-type PTPσ-transfected cells treated with BS_3 chemical crosslinker. Proteins transferred to a membrane were probed with antibodies against the amino-terminal FLAG tag.

D., E. Protein lysates (**W**ild type, C866**L**, C866**S**) were analysed by reducing (D) and non-reducing (E) SDS-PAGE. Proteins transferred to a membrane were probed with antibodies against the amino-terminal FLAG tag.

On non-reducing analysis, a smear (†) is seen in all samples, which runs at the top of the gel and is clearly distinct from the bottom of the sample wells.

Band 1. Full-length PTPσ 1(FL_{166})

Band 2. Extracellular domain from amino terminal cleavage (E_{81})

Band 4. Extracellular domain from carboxy-terminal cleavage (E_{90})

4.3.2: Size-fractionation by rate-zonal centrifugation

The finding that wild type and cysteine substituted PTPσ were indistinguishable under these conditions was surprising. A first explanation might be that PTPσ does not dimerise and that the smear seen is an artefact of non-reducing analysis probably mediated by aggregation of non-blocked cysteine residues during denaturation. However, the majority of receptors studied form multimers and the formation of disulphide cross-linked dimers by other cysteine-substituted RPTPs make it unlikely that PTPσ is an exception to this (Jiang et al., 1999). Alternatively, the introduced cysteines might not be located in a suitable orientation for disulphide formation. However in the case of most multimeric receptors including RPTPs, the transmembrane region is implicated in dimer formation and the transmembrane domain of each subunit is usually closely approximated (Constantinescu et al., 2001; Chin et al., 2005; Gadd and Clevenger, 2006; Lin et al., 2006). If PTPσ dimerises the range of juxtamembrane cysteine substitutions should allow at least some of the mutated proteins to form disulphide bonds. Lastly even if the cysteines are in a suitable location, endogenous PTPσ might compete for dimer formation with the exogenously expressed form. This would create mixed dimers unable to form disulphide bonds. This also is unlikely given the high level of mutant protein expression. Given that it is likely that PTPσ dimerises, what is preventing the detection of disulphide cross linked PTPσ dimers in the juxtamembrane cysteine mutants and what is causing smear formation in all the samples making wild type and mutant indistinguishable?

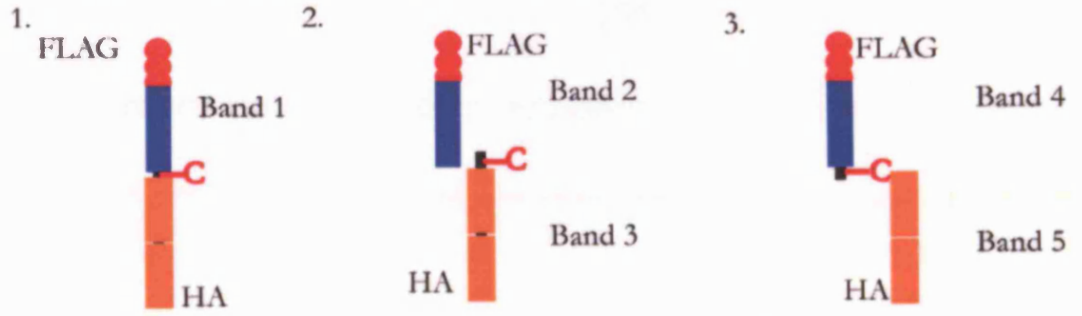
Alternatively, disulphide cross linked dimers may form in the cysteine mutant experiments but be undetectable under these conditions. Disulphide cross-linked dimers might be unstable during lysis, storage or analysis. On the other hand, both the hypothetical full-length PTPσ dimer ($2xFL_{166}$ – Mw~330kDa) and hypothetical cleavage fragment/full-length dimers (FL_{166}/E_{90}, FL_{166}/P_{85} – Mw~250kDa) might be too large to resolve satisfactorily using 6% SDS-PAGE gels. Failure to separate these bands from each other and the full-length PTPσ protein (FL_{166}) would result in a smear between the FL_{166} band and the hypothetical $2xFL_{166}$ band. Lower percentage gels might resolve the dimers better but would fail to retain some of the smaller fragments. However, although these observations explain the presence of a smear rather than discrete bands in the cysteine mutant lanes, they do not explain why the migration patterns of wild type and the cysteine mutants look similar. The high molecular weight smears seen in all samples suggest that disulphide bonds might form as well as break down during electrophoresis or sample handling. Finally, disulphide bonds might form during sample handling or as the proteins

concentrate at the stacking-resolving gel interface during electrophoresis. Such bonds must be mediated by one of the 20 cysteine residues present in wild type PTPσ. These residues are a very unlikely site of physiological disulphide bond formation. The extracellular cysteines already participate in disulphide bonds; stable disulphide bonds are unusual in the reducing intracellular environment and the strikingly conserved transmembrane domain cysteine is not required for high molecular weight smear formation under these conditions. (Figure 4.6 D., E.). However, any of these residues might form artefactual disulphide bonds during sample handling or electrophoresis, which is a likely explanation for the high molecular smear seen in the wild type lanes.

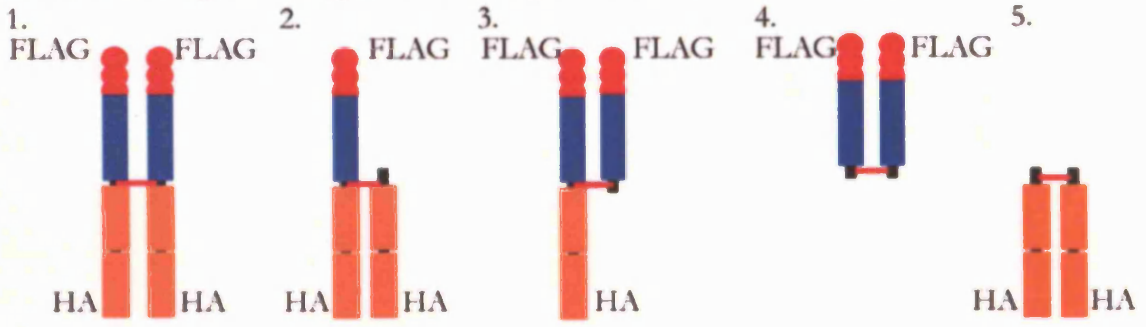
Rate-zonal centrifugation of protein samples separates proteins on the basis of their size. Larger proteins suffer greater drag forces as they pass down a density gradient. The long period of the separation should allow subunit exchange and dimer formation to reach an equilibrium. This may yield improved resolution when compared to SDS-PAGE analysis. The centrifugation step would also have the effect of diluting the sample rendering spurious interaction less likely. Lastly, even if complexes separate during electrophoresis, fragments from larger complexes should be found in fractions higher up the centrifuge than those from small complexes. This should allow better investigation of the high molecular weight smears seen following non-reducing analysis of wild type PTPσ.

Protein samples from cells expressing wild type or cysteine-substituted PTPσ were separated by rate-zonal centrifugation and the resulting fractions were analysed by SDS-PAGE. Total protein staining of a proportion of the fractions showed that some degree of size separation was achieved by rate-zonal centrifugation. (Figure 4.7 C) Following rate zonal centrifugation, only the species previously described (FL_{166}, $E_{90/81}$) were seen on SDS-PAGE analysis of lysates containing wild type PTPσ (Figure 4.7 D). On the other hand, cysteine substituted mutant (839-845C) PTPσ formed two additional bands at approximately 250kDA and >300kDa (Figure 4.7 E). These may correspond to the dimeric FL_{166} species ($2xFL_{166}$) and one or more of the predicted heterodimers. It can be imagined that if these bands were condensed into a single lane, then a smear rather than a series of discrete bands might be formed. It appears therefore that the cysteine substitutions do allow the isolation of dimeric disulphide bonded PTPσ species using rate zonal centrifugation. However, the technique was labour intensive and exhibited significant interexperimental variability as the facilities required to pour reproducible gradients were not available. Therefore, it was important to develop an alternative experimental technique or to optimise the non-reducing SDS-PAGE analysis of cell lysates.

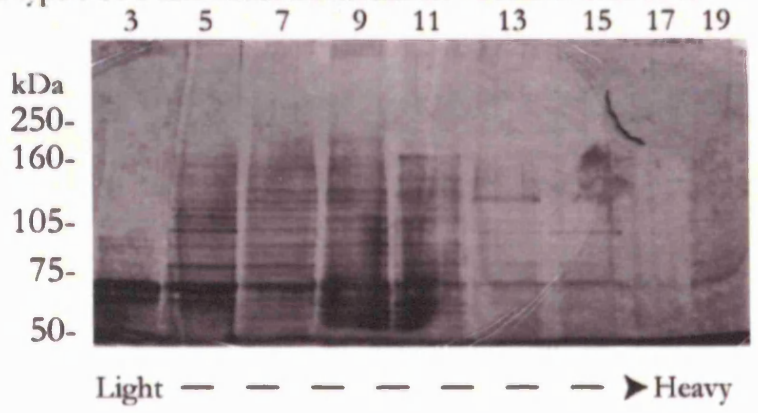
A. Schematic diagram of PTPσ cleavage fragments



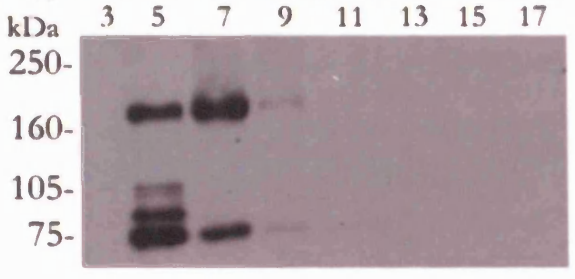
B. Schematic diagram of hypothetical disulphide cross-linked PTPσ dimers



C. Wild type PTPσ rate zonal fractionation - brilliant blue stain



D. Wild type PTPσ rate zonal fractionation - FLAG detection



E. P841C PTPσ rate zonal fractionation - FLAG detection

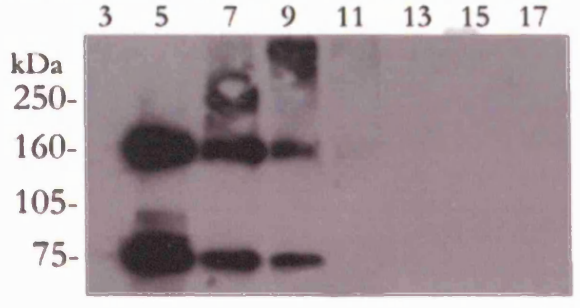


Figure 4.7: Rate-zonal fractionation of wild-type and P841C PTPσ

A. Schematic diagram of PTPσ proteins to show tag locations, expected cleavage products and the locations of the ectopic cysteines (Red C)
B. Schematic diagram of PTPσ proteins to show the hypothetically possible disulphide cross-linked PTPσ dimers. (1) $2xFL_{166}$ – Mw~330; (2) FL_{166}/P_{85} – Mw~250; (3) FL_{166}/E_{90} – Mw~255; (4) $2xE_{90}$ – Mw~180; (5) $2xP_{85}$ – Mw~170.

Cell lysates from HEK-293T cells transfected with wild type or P841C PTPσ were fractionated by rate zonal centrifugation and analysed by non-reducing SDS-PAGE.

D. Silver stain analysis of alternate fractions 3-19
E. Flag (Amino-terminal) detection of alternate fractions 3-17 from wild type lysates
F. Flag (Amino-terminal) detection of alternate fractions 3-17 from P841C mutant lysates. Similar results were seen with the remaining cysteine mutants (839-845C).

Band 1. Full-length PTPσ 1(FL_{166})

Band 2. Extracellular domain from amino terminal cleavage (E_{81})

Band 4. Extracellular domain from carboxy-terminal cleavage (E_{90})

4.3.3: Non-reducing one-dimensional SDS-PAGE with cysteinyl-modifying reagents

If the high molecular weight smears were due to artefactual disulphide formation post-lysis or if they represented background levels of aggregation after destruction of specific disulphide bonded dimers then a clearer picture should be seen if samples are prepared in an excess of an irreversible cysteine modifying reagent (e.g. N-ethylmaleimide (NEM) or iodoacetamide(IAA)). This would have the effect of blocking all exposed cysteine residues not involved in disulphide bond formation and preventing artefactual disulphide bond formation. In addition, the enzymes capable of reducing disulphide bonds all rely on cysteine residues and so cysteine-modifying agents also irreversibly inactivate these enzymes.

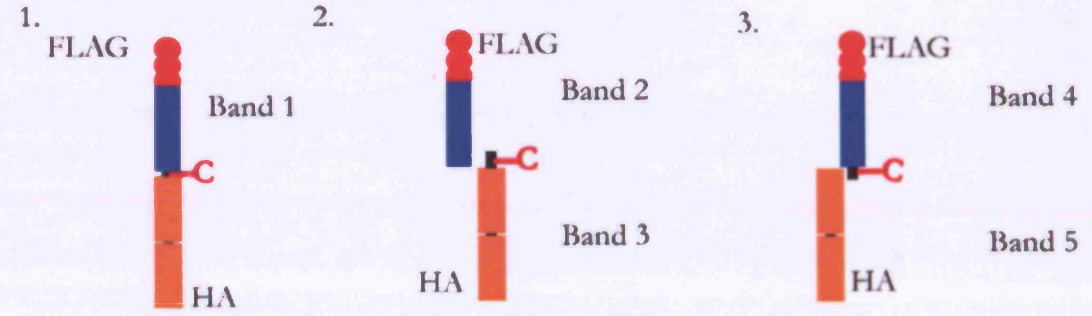
Non-reducing analysis of wild type and cysteine substituted PTPσ from lysates containing either NEM (Data not shown) or IAA clearly show disulphide crosslinking of cysteine substituted PTPσ proteins. In contrast, very little if any disulphide crosslinked wild-type PTPσ was detectable (Figure 4.8), suggesting that the high molecular weight smear described above does represent artefactual post-lysis disulphide mediated aggregation. Analysis of non-reduced lanes containing the cysteine-substituted protein with the amino-terminal FLAG antibody reveals, in addition to the four bands (1,2,4 and 6) previously described on reducing analysis, three reduction sensitive bands (8, 9 and 10), which run at approximately 180 and 300kDa respectively (Figure 4.4; Figure 4.8). The band that runs at approximately 180kDa (Band 8) was reported above (Section 4.2). This band is more prominent on non-reducing analysis than on reducing analysis and its appearance is associated with a decrease in the relative amount of the E_{90} band. Moreover, no corresponding band is seen on HA detection of non-reducing gels, suggesting that this species contains only FLAG-tagged PTPσ ectodomains. Analysis of the same gels with the carboxy-terminal antibody shows three new reduction sensitive bands (bands 9,10,11) in addition to those (bands 1,3,5) described previously (Figure 4.4; Figure 4.8).

The characteristics of the 300kDa species suggest that they might represent disulphide cross-linked dimers of full-length PTPσ (i.e. $2xFL_{166}$). The features of the 180kDa species (Band 8) seen on FLAG analysis and the 170kDa band (Band 11) seen on HA analysis suggest that these correspond to dimeric forms of the PTPσ cleavage products, $2xE_{90}$ and $2xP_{85}$, respectively. On HA detection, it is striking that the P_{85} band is relatively more dimeric than the E_{90} band and that almost all P_{85} is present in the dimeric state. There is no evidence of a heterodimeric interaction between full-length and cleaved

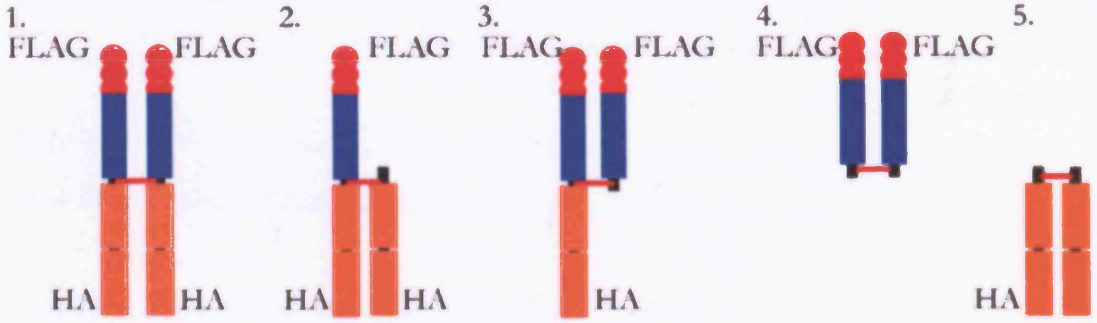
PTPσ (FL_{166}/E_{90} or FL_{166}/I_{85} dimers), which would result in a band intermediate in size between $2xFL_{166}$ and $2xE_{90}/2xP_{85}$ of approximately 250 kDa. However, the gel area corresponding to the $2xFL_{166}$ appears to contain two bands in many of the samples. The additional band is too large to represent the heterodimer referred to above. The nature of one of these two bands is unclear but may reflect some heterogeneity in PTPσ post-translational processing resulting in slight size differences in the dimer. It therefore appears that a significant amount of PTPσ is present in the dimeric form and that all three of the PTPσ species (FL_{166}, E_{90} and P_{85}) that contain the ectopic cysteines form disulphide bonds. However, formal identification of these novel species was not possible using these methods and so two-dimensional reducing/non-reducing electrophoresis was used to identify the components of each species (4.3.4).

The nature of the bands seen in lysates containing the D837C mutant is interesting (Figure 4.4, Figure 4.8). The pattern of D837C bands seen on reducing gel analysis has previously been described. Briefly, little full-length protein is seen and generally only the products from a single cleavage event are detectable. These cleavage fragments are different in size to those normally seen suggesting the use of an alternate cleavage site. On non-reducing analysis, novel bands are seen at approximately 180kDa and 300kDa with the FLAG antibody. This suggests again that the 180kDa species (band 8) contains only cleaved PTPσ ectodomains. However, no additional bands are seen on HA detection of the D837C mutant protein, in keeping with the predicted cleavage point for PTPσ, which would place the disulphide crosslinking site in the extracellular rather than the intracellular fragment predicted to produce the D837C peptides.

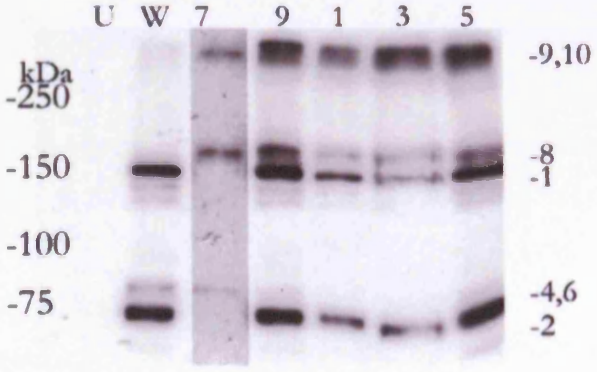
A. Schematic diagram of PTPσ cleavage fragments



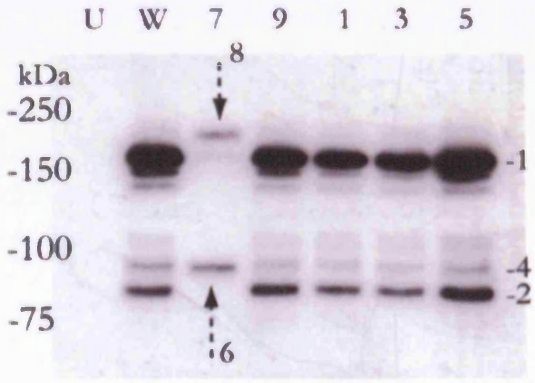
B. Schematic diagram of hypothetical disulphide cross-linked PTPσ dimers



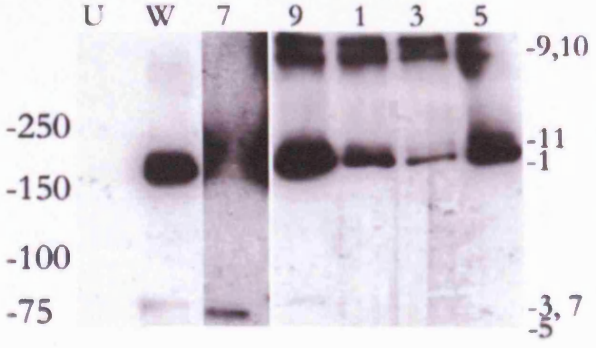
C. FLAG Detection (Non-reduced)



(Reduced - from Fig. 4.4)



D. HA Detection (Non-reduced)



(Reduced - from Fig. 4.4)

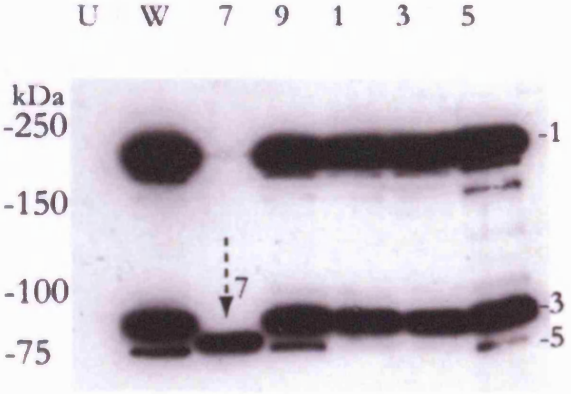


Figure 4.8 Comparative SDS-PAGE analysis of wild-type and cysteine-substituted PTPσ under non-reducing conditions in the presence of iodoacetamide

A. Schematic diagram of PTPσ proteins to show tag locations, expected cleavage products and the locations of the ectopic cysteines (C)

B. Schematic diagram of PTPσ proteins to show the hypothetically possible disulphide cross-linked PTPσ dimers. (1) $2xFL_{166}$ – Mw~330; (2) FL_{166}/P_{85} – Mw~250; (3) FL_{166}/E_{90} – Mw~255; (4) $2xE_{90}$ – Mw~180; (5) $2xP_{85}$ – Mw~170.

Protein lysates (**U**ntransfected, **w**ild type, D83**7**C, P83**9**C, P84**1**C, P84**3**C, P84**5**C) were analysed by non-reducing SDS-PAGE. Figure 4.4 and Figure 4.8 were run in parallel analysing the same samples under reducing and non-reducing conditions respectively. Samples from D837C mutants required longer exposures for detection.

Proteins transferred to a membrane were probed with antibodies against the amino-terminal FLAG (C) or carboxy-terminal HA tags (D). The right hand panels show reduced gels for comparison (Figure 4.4).

A band (8) is visible at approximately 180kDa in all the cysteine mutant lysates but not in the wild type lysates. A similar novel band (11 – approximately 170kDa) is seen in HA detections of these samples.

Band 1. Full-length PTPσ 1(FL_{166})
Band 2. Extracellular domain from amino terminal cleavage (E_{81})
Band 3. Phosphatase domain from amino-terminal cleavage (P_{85})
Band 4. Extracellular domain from carboxy-terminal cleavage (E_{90})
Band 5. Phosphatase domain from carboxy-terminal cleavage (P_{75})
Band 6. Extracellular domain fragment from D837C mutant (E_{837})
Band 7. Phosphatase domain fragment from D837C mutant (P_{837})
Band 8. Putative disulphide crosslinked dimer of band 4 (FLAG positive, HA negative)
Band 9. Putative disulphide crosslinked dimer of band 1 (FLAG positive, HA positive)
Band 10. Putative disulphide crosslinked dimer of band 1 (FLAG positive, HA positive)
Band 11. Putative disulphide crosslinked dimer of band 3 (FLAG negative, HA positive)

Immunofluorescent and SDS-PAGE analysis revealed no difference between wild-type and the 839-45C cysteine-substituted PTPσ proteins. On the other hand, the D837C mutant underwent a distinct form of post-translational processing from all the other mutants. The data shown in chapter 3 suggest that, in an HEK-293T overexpression system, distinct patterns of subcellular localisation occur between uncleaved PTPσ and its cleavage products. Therefore, perturbation of the normal cleavage events might alter the normal distribution of cleaved and uncleaved protein. As full-length protein and some of the cleaved protein species are both labelled with the epitope tag it is not possible to detect their localisation independently using immunofluorescence except by comparing overlapping patterns of detection with antibodies that detect amino- and carboxy-terminal epitopes respectively. In chapter 3, a method was described to determine whether PTPσ species are located at the cell membrane or inside the cell. Limited cell trypsinisation prior to lysis results in the digestion of extracellular but not intracellular protein. Furthermore, carrying out this experiment under non-reducing conditions allowed dimeric and monomeric PTPσ to be followed separately.

Analysis of the cysteine-substituted mutants using limited cell-surface trypsinisation likewise revealed no differences between wild-type and 839-845C substituted PTPσ (Figure 4.9). The effect of cell surface trypsinisation on wild type PTPσ was discussed in detail above and will not be repeated here (3.2.2). Using the FLAG epitope tag, under non-reducing conditions wild type PTPσ produced three major bands, FL_{166}, E_{81} and E_{90}. Under non-reducing conditions, the 839-845C PTPσ mutants exhibited all the expected bands on FLAG detection. High molecular weight bands 9 and 10 are readily detected as is non-dimeric full-length PTPσ (FL_{166}). The 180kDa band (8) is detected weakly in all lanes and the two cleaved fragments are clearly present. As with wild type PTPσ, the E_{81} fragment of the mutant PTPσ proteins alone appears to be resistant to trypsin treatment Under the same conditions, four bands are detected in cells expressing the D837C mutant: as described above. However, following digestion, preservation of E_{81} is also seen in D837C, which predominantly expresses an E_{90}-like fragment prior to digestion.

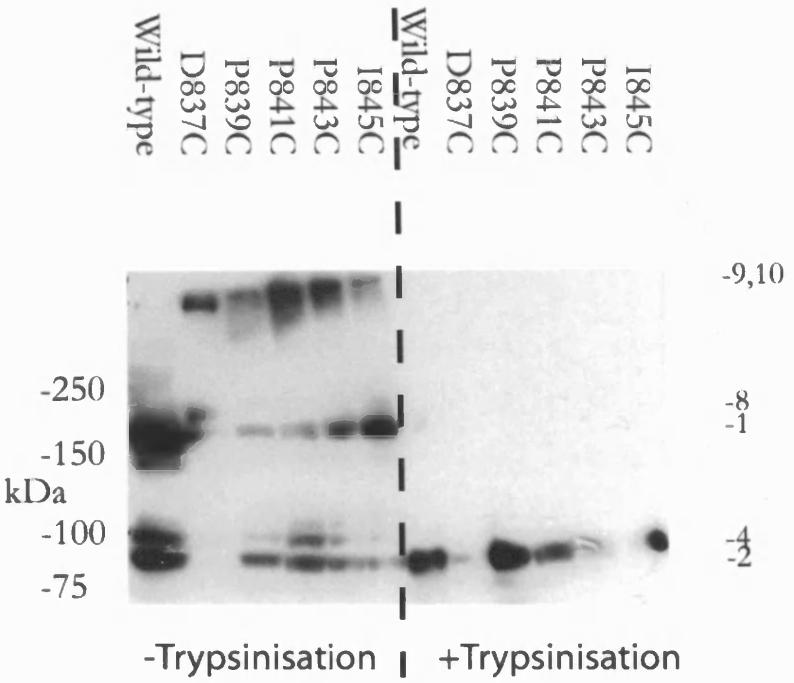


Figure 4.9 Localisation of wild-type and cysteine substituted PTPσ proteins by limited cell surface trypsinisation

Non-reducing SDS-PAGE analysis of lysates from HEK-293T cell cultures transiently transfected with wild-type or mutant PTPσ. Prior to lysis cells were incubated for fifteen minutes in digestion buffer with (Right hand lanes) or without trypsin (Left hand lanes). Lysates were detected with the FLAG antibody. PTPσ species are labelled at the right of each gel image (FL_{166} and $E_{90/81}$).

Band 1. Full-length PTPσ 1(FL_{166})

Band 2. Extracellular domain from amino terminal cleavage (E_{81})

Band 4. Extracellular domain from carboxy-terminal cleavage (E_{90})

Band 8. Putative disulphide crosslinked dimer of band 4 (FLAG positive, HA negative)

Band 9. Putative disulphide crosslinked dimer of band 1 (FLAG positive, HA positive)

Band 10. Putative disulphide crosslinked dimer of band 1 (FLAG positive, HA positive)

4.3.4: Two dimensional non-reducing/ reducing SDS-PAGE

Non-reducing SDS-PAGE analysis of lysates from cells expressing wild type or cysteine substituted PTPσ shows that cysteine substituted PTPσ forms disulphide bonded oligomers. However, these high molecular weight bands might also represent non-specific aggregates of PTPσ due to overexpression or disulphide bonded oligomers of PTPσ with other proteins. However, under non-reducing conditions, several bands appear at the predicted sizes for dimeric PTPσ species. In addition, the relative size differences between the species are not constant. Therefore, if PTPσ is forming specific oligomers with other proteins then each additional PTPσ containing species must interact with a different protein. In conclusion, PTPσ is most likely to be forming specific disulphide bonded homodimers in these experiments.

It is important to resolve which of the possible explanations for the high molecular bands is in fact the case. Two-dimensional gel electrophoresis can resolve the components of each band. The samples were initially run through an SDS-PAGE system under non-reducing conditions. The sample lane was then carefully excised and incubated in an equilibration buffer in either the presence or absence of reducing agents. Complete reduction of the intramolecular disulphide bonds was not possible and improved reduction was associated with increased protein diffusion through and out of the gel. Resolution and retention were already poor due to diffusion during equilibration and at the gel interfaces. The sample lane was then applied to the top of a second gel and run perpendicularly to the direction of the first gel. If the equilibration step is non-reducing then the PTPσ peptides will run at the same rate in the second as in the first gel and so will form spots in a diagonal line that runs at approximately 45°. Under reducing conditions, any disulphide crosslinked dimers will be broken into their monomeric constituents and so will run faster in the second gel than in the first and so will appear below this diagonal line. (Figure 4.10)

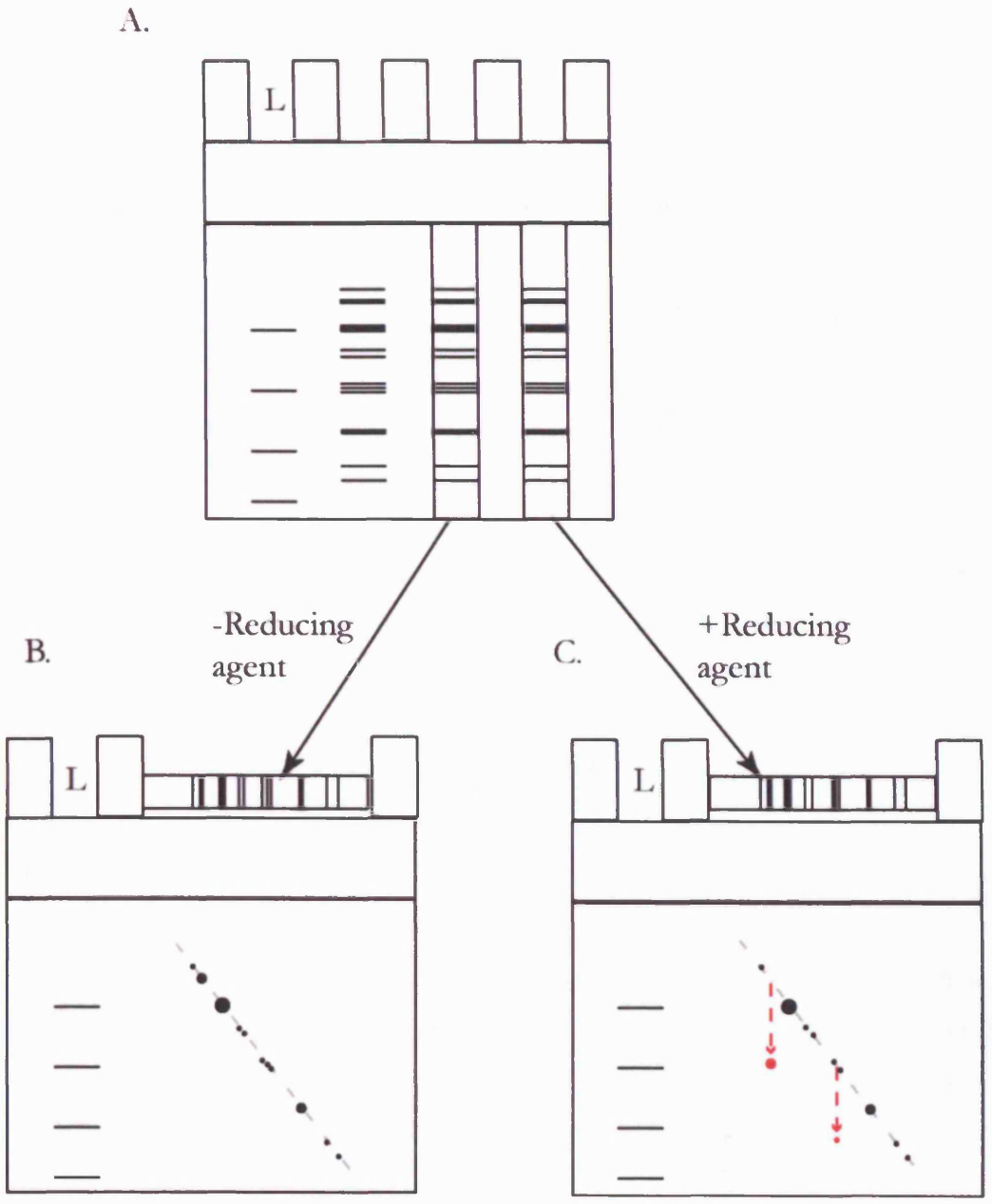


Figure 4.10 Schematic diagram to show two-dimensional gel system

A. Conventional 6% SDS-PAGE gel analysis of PTPσ containing lysates. Lanes containing samples are excised and incubated in equilibration buffer before being applied to the top of second dimension gels at right angles to the direction of migration in the first dimension.

B. Second dimension after non-reducing equilibration – proteins run at the same rate as in the first dimension and so are found on a 45° line (dotted grey).

C. Second dimension after reducing equilibration – proteins containing intermolecular disulphides (Red) dissociate to their constituent monomers and so run off the diagonal. (L=ladder)

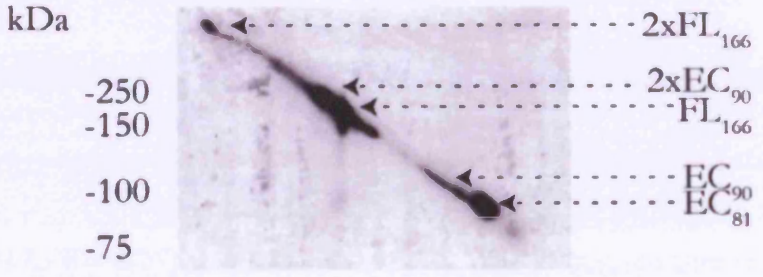
Two-dimensional SDS-PAGE analysis initially under non-reducing conditions then in a perpendicular direction in the presence or absence of reducing agents confirmed that PTPσ forms specific disulphide bonded dimers. Under two-dimensional electrophoresis where both dimensions were non-reducing, the migration pattern is identical to that seen in one-dimensional electrophoresis under non-reducing conditions albeit in a diagonal plane (Figure 4.11A, C). Very little protein migrated off the 45° angle that indicates migration at the same rate in each of the two dimensions. The system was also validated using commercial protein markers to ensure that the process did not affect the migration rate of proteins in the second dimension slice (Data not shown). This confirms that the two-dimensional technique per se does not affect disulphide bonds.

Wild type and all of the cysteine substitutions except D837C were examined using this technique. Wild-type PTPσ showed no significant differences when the spot pattern obtained under the two conditions was compared (Data not shown). Identical results were obtained for each of the remaining mutants (Data not shown). As an example, the experiments using P839C will be described in detail. These experiments confirm that the novel PTPσ species seen on non-reducing analysis of the disulphide crosslinking mutants represent dimeric forms of PTPσ. Therefore, from this point on these species will be referred to by their content (i.e.$2xFL_{166}$) rather than as a numbered band (i.e. band 9). Using the amino-terminal FLAG antibody, the major species seen on non-reducing → non-reducing analysis are dimeric full-length protein ($2xFL_{166}$), dimeric cleaved extracellular fragment ($2xE_{90}$), full-length protein (FL_{166}) and cleaved extracellular domain (E_{81}) (Figure 4.11A). Following reduction of the first dimension gel slice, these bands are still prominent but additional bands are apparent at approximately 160 kilodaltons below the site of the $2xFL_{166}$ spot and at approximately 90 kilodaltons below the $2xE_{90}$ spot (Figure 4.11B). These new spots represent disruption of the disulphide bonded dimeric species and confirm the nature of these species. The size of the dimeric species therefore correlates with the monomeric content and so the oligomers are likely to be PTPσ homooligomers rather than heterooligomers with a range of different proteins. Again, no heterodimeric FL_{166}-E_{90} dimers are apparent and there are no spots corresponding to the dissociation of these intermediately sized oligomers on non-reducing → reducing two-dimensional analysis.

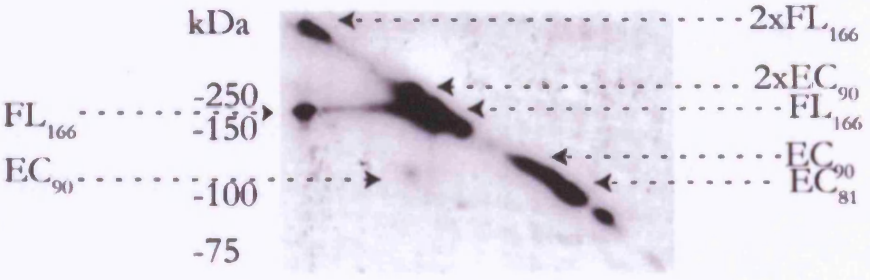
Analysis of two dimensional gels with the carboxy-terminal HA antibody gave corresponding results. The major species seen on non-reducing → non-reducing analysis were dimeric full-length protein ($2xFL_{166}$), full-length protein (FL_{166}) and dimeric cleaved

intracellular fragment ($2xP_{85}$) (Figure 4.11C). On non-reducing → reducing analysis, these spots were still visible but additional spots appeared running at approximately 160 kilodaltons below the $2xFL_{166}$ spot and at approximately 85 kilodaltons running beneath the $2xP_{85}$ spot (Figure 4.11D). These correspond to the monomeric constituents following dissociation of disulphide bonded dimeric $2xFL_{166}$ and $2xP_{85}$ respectively. It is worth noting that the dimeric extracellular and intracellular cleavage products of PTPσ and their constituent monomers run respectively larger or smaller than the monomeric full-length protein. This suggests that the appearance of these bands is not due to artefactual proteolysis of PTPσ during the two-dimensional procedure but reflect the expected differences in the proteins detected with each tag.

A. FLAG - Non-reducing 2nd dimension



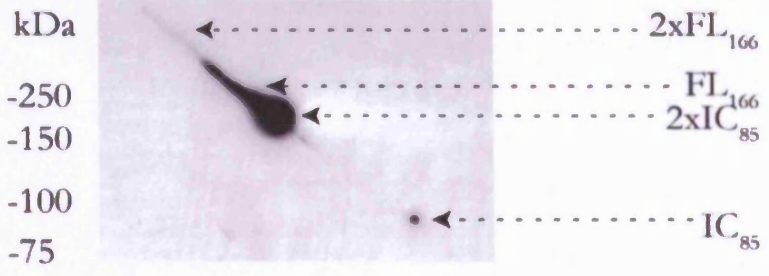
B. FLAG - Reducing 2nd dimension



2nd Dimension

1st Dimension (Non-Reduced)

C. HA - Non-reducing 2nd dimension



D. HA - Reducing 2nd dimension

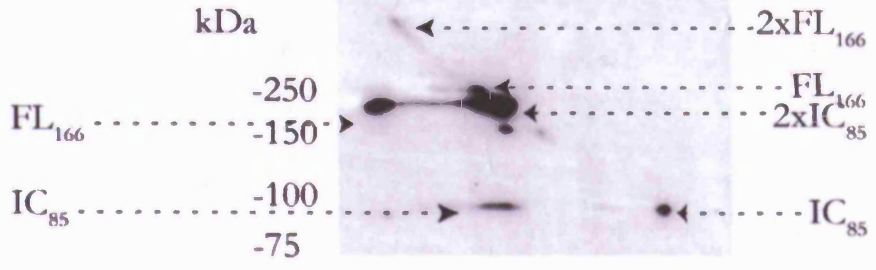


Figure 4.11: 2-dimensional non-reducing/reducing SDS-PAGE analysis of P839C PTPσ.

Cell lysates were analysed as described by non-reducing SDS-PAGE. The sample lanes were excised and incubated in equilibration solution with or without reducing agents as described. The equilibrated sample lanes were then turned 90° and cast horizontally into the top of a second SDS-PAGE gel and analysed as described.

Monomeric and dimeric PTPσ species are labelled at the right of each gel image (FL_{166}, $E_{90/81}$ and $P_{85/75}$). An equal amount of the same batch of lysate (μg total protein) was loaded in each lane.

A. 1st dimension – non-reducing; 2nd dimension – non-reducing. FLAG (Amino-terminal) detection
B. 1st dimension – non-reducing; 2nd dimension –reducing. FLAG (Amino-terminal) detection
C. 1st dimension – non-reducing; 2nd dimension – non-reducing. HA (Carboxy-terminal) detection
D. 1st dimension – non-reducing; 2nd dimension –reducing. HA (Carboxy-terminal) detection.

4.4: Discussion

The work presented in this chapter describes the dimerisation of PTPσ for the first time. A long-range site-directed mutagenesis system was developed and used to introduce ectopic cysteine substitutions into the PTPσ cDNA. These mutations as a whole had no detectable effect on PTPσ localisation or cleavage or on the behaviour of the transfected cells when compared to wild type PTPσ. However, the resolution and heterogeneous expression levels of these transient transfection experiments makes any firm conclusion impossible. This could be addressed by confocal and SDS-PAGE analysis of stably transfected cells expressing PTPσ at more physiological levels. In contrast, the mutation D837C alters the site and the regulation of PTPσ cleavage. If the lysates from cysteine-substituted PTPσ were analysed by SDS-PAGE under non-reducing conditions, disulphide cross-linked dimers were detected. Dimeric species were isolated from all of the PTPσ fragments containing the cysteine residues: FL_{166}, E_{90} and P_{85}. Heterodimeric PTPσ species (e.g. FL_{166}+E_{90}) were not detected. Using two-dimensional electrophoresis, the dimeric full-length PTPσ species was shown to contain only FL_{166} subunits and the dimeric cleavage fragments, only E_{90} or P_{85} subunits.

The large template mutagenesis method developed in this chapter is a straightforward method that will often require the purchase of only a single mutagenic primer. This mutagenic primer can then be used with any suitable primer already held in the laboratory to produce a 300-400bp product. However, the method is still not ideal and the number of mutant colonies recovered is still low. It remains to be seen if site-directed mutagenesis reactions can be converted into exponential amplification reactions by judicious off-setting of the two mutagenic primers either in single-stage or two-stage site directed mutagenesis reactions.

The disulphide bonding experiments provide evidence for a model for PTPσ structure. Dimers of full-length PTPσ and of each of the longer cleavage fragments were detected on non-reducing SDS-PAGE analysis of lysates containing cysteinyl modifying reagents. SDS-PAGE analysis of lysates lacking iodoacetamide or N-Ethylmaleimide only showed diffuse high molecular weight smears and these were seen in lysates from cells expressing either wild-type and or disulphide crosslinking cysteine mutant PTPσ. The significance of these high molecular weight smears in these lysates will be discussed in a subsequent chapter (Chapter 6). Heterodimers of PTPσ full-length protein with cleaved protein were not detected and using this system, it is not possible to exclude the formation

of E_{90}/P_{85} heterodimers as these cannot be distinguished from the homodimeric species which they are very similar to in size. However, there is no evidence that either of these heterodimeric species form.

It is not formally possible at this stage to confirm that the disulphide bonded bands seen on non-reducing SDS-PAGE analysis represent PTPσ dimers or heterodimers of PTPσ with other proteins. However, heterodimers of PTPσ with other proteins are an unlikely explanation as the size difference between the proposed PTPσ full-length dimers and the PTPσ cleavage product dimers is the appropriate size for PTPσ homodimers. If heterodimers are the explanation, then the $2xEC_{90}$, $2xIC_{85}$ and $2xFL_{166}$ species would each represent dimers of specific PTPσ species with distinct interacting partners of different molecular weights. Furthermore the region of interaction for the disulphide bond formation can be restricted to the fragment of protein between the two cleavage sites. In addition, two-dimensional analysis confirms that the $2xFL_{166}$ species contains only full-length protein on reduction and that the $2xEC_{81}$ species releases only E_{81} on reduction. However, the only way to formally confirm that these bands represent homodimeric PTPσ is to immunoprecipitate differentially tagged disulphide crosslinked PTPσ and run two-dimensional electrophoresis and co-immunoprecipitation experiments (Chapter 5). Finally, these experiments do not unequivocably confirm that these species are dimers rather than other higher order multimers of PTPσ. However, wild type protein does not form these species and each cysteine mutant contains only a single ectopic cysteine that can be responsible for the disulphide bonds seen. Therefore, these complexes are almost certainly dimeric.

A very high degree of dimerisation was seen in the disulphide cross-linking experiments. However, little inference can be drawn from this finding, as disulphide cross-linking would be expected to over-represent the level of dimerisation seen in the cell by trapping any dimers that do form. It can be concluded however, that dimerisation is unlikely to be associated with degradation of PTPσ as there was no difference in level between wild type and all of the cysteine substitutions mutants except D837C. The only ways to confirm the physiological level of dimerisation are co-immunoprecipitation of differentially tagged PTPσ or resonant energy transfer techniques using cells expressing PTPσ at physiological levels. Both of these techniques are better suited to comparison of the relative amounts of dimerisation under various conditions. They both tend to underestimate the level of dimerisation due to the expected forms of dimer formed (i.e. subunits A+B → AA, AB, BA, BB); at most, half of all dimers formed will be detected

using these techniques. Quantitative fractionation of native PTPσ complexes might be able to resolve the question but would be technically difficult. It was noted that significant amounts of disulphide crosslinked protein remained on reducing SDS-PAGE analysis. This may reflect a resistance to reduction or the reformation of disulphide bonds during electrophoresis despite the inclusion of cysteinyl modifying reagents in these samples. Resistance of the juxtamembrane disulphide bond to reduction is a prominent feature of these proteins and probably indicates that the juxtamembrane bond is deeply buried and relatively inaccessible to reducing agents. A similar pattern is seen in the relative accessibility of the various insulin receptor disulphide bonds to reduction (Massague and Czech, 1982). Unfortunately, it is not possible to include reducing agents in the gel substance of acrylamide gels, as this would interfere with polymerisation.

Assuming that the novel species detected following disulphide crosslinking do represent PTPσ dimers, their relevance is still open to debate for the following reasons. They may be an artefact of the high levels of overexpression achieved in these experiments and protein overexpression has previously been shown to induce aggregation of wild type receptors (Di Fiore et al., 1987; Di Marco et al., 1990; Maru et al., 1990; Brandt-Rauf et al., 1990). However, it is impossible to perform the disulphide cross-linking or co-immunoprecipitation experiments necessary to answer this question in vivo using endogenous levels of PTPσ expression. However, stable cell lines expressing endogenous levels of PTPσ might be created to answer this question. Alternatively, the disulphide bonds themselves might be necessary and sufficient for dimerisation. The requirement for disulphide bonds in PTPσ dimerisation can readily be determined by co-immunoprecipitation experiments.

The PTPσ species, FL_{166} and $E_{90,}$ were shown to be predominantly located on the cell surface whereas the E_{81} species was predominantly intracellular (Chapter 3). This also applied to the dimeric forms of FL_{166} and E_{90}, which were mostly cell surface located. This is in conflict with some of the available literature. Cell-surface labelling suggests that only cleaved protein is expressed on the cell surface and pulse chase data suggests that full-length PTPσ is rapidly cleared and that cleaved protein is rapidly degraded (Streuli et al., 1992). However, full-length PTPσ (and other type II RPTPs) can be detected inside the cell both physiologically and following cellular treatments (Aicher et al., 1997). The most parsimonious explanation for these contrasting observations is that PTPσ is expressed on the cell surface in the full-length form (both monomeric and dimeric),which is rapidly cleaved at the amino-terminal site. Both monomeric and dimeric forms of the protein

appear to be susceptible to cleavage with internalisation of the resulting fragment (Chapter 3). Possibly, previous work only detected cleaved protein on the surface as by the time the cells were analysed at the end of labelling most of the labelled protein had been internalised following cleavage. Most E_{81} is intracellular therefore amino-terminal cleavage of dimers may affect both peptide chains. Alternatively, cleavage of a single subunit in a formed dimer may result in the immediate shedding of the cleaved extracellular domain. Furthermore, it was not possible to detect heterodimers comprising any cleavage fragment disulphide bonded to a full-length PTPσ molecule. There are several possible explanations for this finding. Firstly, only full-length PTPσ may be able to form dimers. The dimeric cleavage fragments might represent the continued association of the fragments that result from the proteolytic cleavage of a dimeric species. Alternatively, there is some data that suggests that the full-length and cleaved PTPσ species occupy different parts of the cell and so might not be able to interact for this reason. Several stimuli can drive PTPσ cleavage which is associated with redistribution of PTPσ (Aicher et al., 1997). However, the relationship between cleavage and redistribution is unclear. It can be noted that as both full-length and cleaved PTPσ have dimeric forms, it is unlikely that dimerisation *per se* regulates cleavage or conversely, is regulated by cleavage.

The tools used in this thesis do not allow the identification of the results of dimer cleavage at the amino-terminal site. No P_{85}-FL_{166} heterodimers are present as remarked above but any dimeric E_{81} species are undetectable as they lack the cysteine residues necessary for disulphide formation. However, dimer cleavage at the carboxy-terminal site results in the $2xEC_{90}$ staying on the cell surface. Eventually the $2xEC_{90}$ form is either internalised or shed as long-term treatment with agents that induce cleavage at the carboxy-terminal site results in complete extracellular domain internalisation. There is not sufficient data available to comment on the localisation of the cleaved intracellular domain fragments in either monomeric or dimeric form but previous studies suggest that it is internalised with the extracellular domain (Aicher et al., 1997).

Previously published models for type IIa RPTP cleavage suggest that the amino-terminal cleavage event occurs approximately 100 amino acids from the cell membrane via a subtilisin-like protein convertase (PC) (Streuli et al., 1992; Jiang et al., 1993; Yan et al., 1993; Serra-Pages et al., 1994; Pulido et al., 1995a; Campan et al., 1996; Cheng et al., 1997). The prevailing consensus is that this cleavage event is a constitutive part of type IIa RPTP maturation, occurring immediately after a conserved dibasic PC recognition motif. The model further suggests that carboxy-terminal cleavage is an inducible-event occurring close

to the transmembrane domain that is catalysed by a matrix metalloproteinase or "a distintegrin and metalloprotease" (ADAM) family protease (Serra-Pages et al., 1994; Aicher et al., 1997; Ruhe et al., 2006; Anders et al., 2006). These enzymes appear to have significant flexibility as to their recognition sequence (Serra-Pages et al., 1994; McFarlane, 2003; White, 2003; Huovila et al., 2005; Malemud, 2006). Cleavage at this site following amino-terminal cleavage is thought to allow shedding of the E_{81} fragment and is associated with a redistribution of the remaining detectable intracellular domain to the inside of the cell.

In agreement with the data presented above (Chapter 3), all PTPσ proteins except the D837C mutation showed significant amounts of cell surface located E_{90}. This can only be produced by carboxy-terminal cleavage in the absence of amino-terminal cleavage. This suggests that amino-terminal cleavage is not a constitutive part of PTPσ maturation nor need it occur before carboxy-terminal cleavage. However, it may be suggested that expression of full-length and E_{90} are artefacts of overexpression reflecting the limited capacity of the conventional PTPσ processing pathway. This subject has been discussed above (Chapter 3 and this chapter). Furthermore, an E_{81}-like species was seen in lysates from trypsinised cells expressing D837C PTPσ, which do not express significant levels of an E_{81} species normally. The E_{81}-like species seen might be derived from digestion of either the 180kDa band (8) or the limited amounts of full-length D837C PTPσ seen with this mutant. It was not possible to determine the origin of this species using the techniques employed in this chapter.

Of the five mutations, only the D837C mutation has a detectable effect on PTPσ processing. It is difficult to explain why the D837C mutation, which is located furthest from the predicted site of carboxy-terminal cleavage, is the only mutant to affect carboxy-terminal cleavage. The D837C mutation increases the cleavage rate of full-length PTPσ as indicated by the disproportionate decrease in the amount of full-length as compared with cleaved D837C PTPσ protein. Cleavage of the D837C mutant PTPσ protein occurs at a site that is slightly amino-terminal to the canonical carboxy-terminal cleavage site. This is unlikely to reflect cleavage by the amino-terminal PC enzyme as this site does not contain the dibasic consensus site required by such enzymes. This generated a single extracellular domain band that was slightly smaller than E_{90} and a single intracellular domain band that was slightly larger than E_{81}. Therefore, the ectopic cleavage site in the D837C mutant protein must be located carboxy-terminal of the amino-terminal cleavage site and amino-terminal of the carboxy-terminal cleavage site. However, as the P_{837} fragment is not able to

form disulphide bonded dimers but the E_{837} fragment can, the cleavage site must be between the carboxy-terminal cleavage site (P841/I842) and the D837C mutation. This only leaves a small window of 4 amino acids for the cleavage site to move into. This is probably too small a change to produce the size shift that is detected from $E_{90/81} \rightarrow E_{837}$ and from $P_{85/75} \rightarrow P_{837}$ even if cleavage shifts as far amino-terminal as these constraints allow, that is if cleavage occurs at 837/838 or 838/839.

An alternative explanation is that the PTPσ cleavage site previously identified in A431 cells is not the same site that is utilised in HEK-293T cells overexpressing cPTPσ1 (Aicher et al., 1997). It must also be noted that this site was identified following cleavage induced by TPA, which may not occur at the same site as PTPσ carboxyl terminal cleavage in the resting state. This is supported by the failure of the mutations P841C and P843C to affect cleavage and the lack of any difference in the pattern of disulphide crosslinked species produced by the P839C and P845C mutations, which are predicted to be on alternate sides of the cleavage site. It must be noted however, that the recognition site for carboxyl-terminal cleavage exhibits significant sequence flexibility (Serra-Pages et al., 1994). The size shift detected is approximately 1kDa in size, and as such represents approximately ten amino acids. Assuming that carboxy-terminal cleavage of the wild type protein occurs at the same site as in the 839-45C mutants, then carboxy-terminal cleavage must be occurring C-terminal to 845C, moving the potential cleavage site significantly closer to the membrane, suggesting that intramembrane proteolysis may play a role in PTPσ cleavage. The D837C mutant in this model might affect PTPσ cleavage by a conformational effect rather than by direct disruption of the recognition site. Other ways in which this effect might be mediated include relocalisation of the mutant protein resulting in cleavage by a different protease.

Although the D837C mutant does undergo proteolytic cleavage, the resulting E_{837} fragment is not shed and the level of PTPσ is lower in the D837C mutant than in any other PTPσ protein. This may be the result of increased cleavage of the full-length form or increased stability of the cleaved form with or without changes in PTPσ expression levels. It will be interesting to see if shed D837C mutant protein can be detected following protein kinase C activation by induction with phorbol ester (Chapter 6). If the ectopic cleavage event merely alters the balance of factors controlling PTPσ fate, then boosting the endogenous shedding signal might allow shed E_{837} to be detected. Experiments of this kind will reveal the relationship between the two cleavage events, shedding and PTPσ function. Most importantly, if it is confirmed that the D837C mutant is more rapidly cleaved than

wild type PTPσ but that despite this it is not shed then this might suggest that cleavage is not a cell surface phenomenon.

Lastly, there is a relative paucity of the full-length PTPσ form when compared to the amount of cleaved product in cells expressing the D837C mutant protein. This might be due to increased stability and retention of the cleavage products, for example due to a decrease in shedding. Alternatively, the stability or the susceptibility of full-length D837C protein to proteolytic cleavage may be altered with or without accompanying changes in PTPσ expression level.

The current model of type II RPTP processing does not fit all the available data. An alternative hypothesis might be that full-length protein is in fact an active receptor, which is localised to the plasma membrane. The apparent difference in protein cleavage might not reflect differences in PTPσ processing but rather a difference in the eventual fate of the cleavage fragments. Several groups have followed type II RPTP processing by pulse-chase experiments. No group has dissected the two major events in type II RPTP processing, namely cleavage and degradation and widely differing results have been obtained in different cell types irrespective of whether expression is endogenous or driven by transfection. However, all studies agree that type II RPTPs are cleaved rapidly, typically within 30 minutes of translation. The discrepancy occurs over the time for which the cleaved PTP remains in the cell. The detection of large amounts of cleavage product in many tissues might reflect the detection of free extracellular domains. These might be bound either to the cell surface or inside cells. A number of mutations have been identified that interfere with PTPσ-ligand interactions. It would be relatively straightforward to determine the cleavage pattern of full-length PTPσ expression constructs containing the ligand binding site mutations.

The results in this chapter are similar to those obtained with PTPα and suggest that dimerisation is a common feature of the RPTP family although as PTPα has a smaller extracellular domain than PTPσ, it may not be regulated in the same way (Jiang et al., 1999; Jiang et al., 2000; Tertoolen et al., 2001). However, it is difficult to determine what the effect of PTPσ dimerisation is on catalytic activity. Disulphide cross-linked dimers can be separated only by using reducing agents, which themselves have a marked effect on the activity of cysteinyl-based tyrosine phosphatases. The non-reducing conditions required to maintain the disulphide cross-linked dimers inhibit enzyme activity and the reducing conditions required to separate the disulphide cross-linked dimers into monomers increase

enzyme activity. Therefore, the effect of dimerisation *per* se on enzyme activity would be extremely difficult to determine using the cysteine cross-linked dimers. Moreover, it would be difficult to determine whether the effect seen was representative of the physiological situation or an artefact derived from the cysteine mutations. Having demonstrated that PTPσ dimerises, it will be important to determine the effect of PTPσ dimerisation on its activity and cellular function and to identify factors that affect the proportion or orientation of PTPσ dimers.

Chapter 5: Co-immunoprecipitation of PTPσ oligomers

In chapter four, PTPσ dimerisation was demonstrated using disulphide crosslinking, following the introduction of a number of ectopic cysteine residues. However, all the PTPσ species containing an ectopic cysteine were found to dimerise, which might suggest that the cysteine residue itself is inducing this interaction. That is the disulphide bridges might cause rather than reflect dimerisation. It is important therefore to determine whether dimeric PTPσ can be isolated using a different method with PTPσ protein that is wild type with respect to the cysteine substitutions. In this chapter, the coimmunoprecipitation of differentially tagged wild type PTPσ is described. The results agree with those seen using disulphide crosslinking and strongly support a model for PTPσ function in which significant amounts of PTPσ protein are present in a homo-oligomeric form.

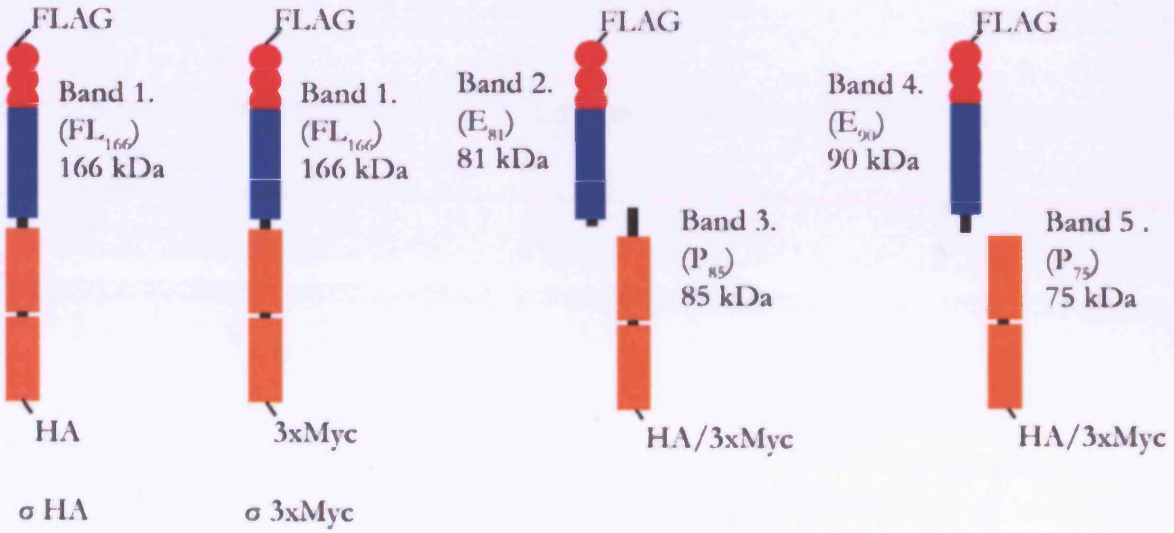
5.1: Co-immunoprecipitation using two distinct intracellular tags

Two different PTPσ constructs were designed such that each had a different carboxy-terminal epitope tag; either an HA tag or a 3xMyc tag (Figure 5.1A). There was no detectable difference in either the localisation or cleavage pattern of these two forms of PTPσ when compared to the other constructs used in this thesis (Data not shown). Cotransfection of HEK-293T cells with these two constructs resulted in the robust expression of the two encoded proteins. Approximately equal levels of each protein were seen in lysates from single- and double-transfected cells (Figure 5.1B, C – lanes 1-4).

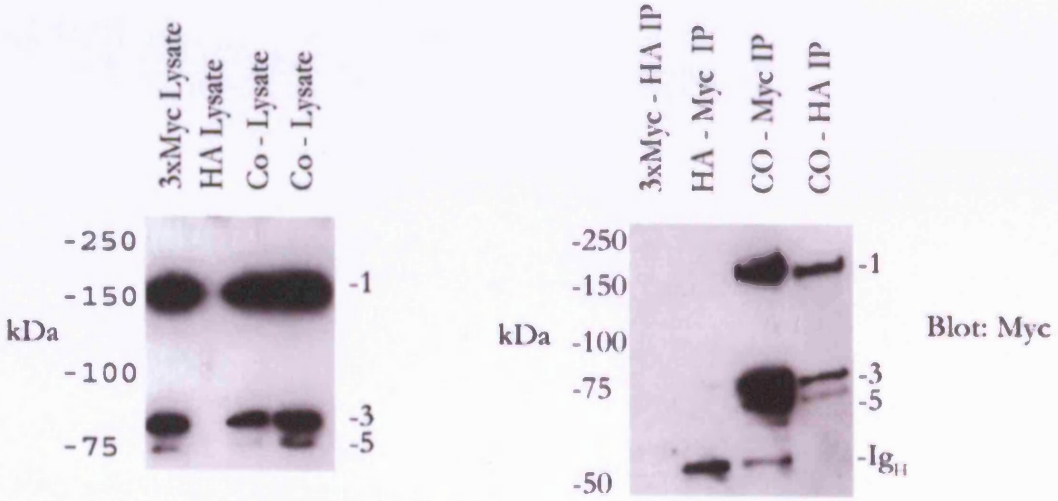
When lysates from cells expressing one of the differentially tagged proteins were immunoprecipitated with an antibody directed against the corresponding tag, PTPσ protein bearing the second tag was readily recovered (Figure 5.1B – lane 8, C – lane 7). All detectable forms of PTPσ were recovered, that is FL_{166} (band 1), P_{85} (band 3) and P_{75} (band 5). On the other hand, when lysates from cells expressing only one of the differentially tagged PTPσ proteins were immunoprecipitated with an antibody directed against the converse tag, no PTPσ protein was recovered (Figure 5.1B, C – lanes 5, 6). Moreover, when immunoprecipitates from cells expressing both lysates were immunoprecipitated with one antibody, this co-immunoprecipitated PTPσ protein labelled with the converse tag. Co-immunoprecipitation is more readily apparent following anti-HA immunoprecipitation of lysates expressing both HA- and 3xMyc tagged lysates. This is due to an unfortunate combination of quantitatively poorer immunoprecipitation with the Myc antibody and

detection with the HA antibody. However, careful examination reveals that all three forms of PTPσ, FL_{166} (band 1), P_{85} (band 3) and P_{75} (band 5), that bear a unique COOH terminal tag coimmunoprecipitate following immunoprecipitation of PTPσ protein bearing the converse tag (Figure 5.1B – lane 8, C – lane 7).

A. Schematic diagram of tagged constructs used in this figure



B. Co-immunoprecipitation of Myc-tagged PTPσ with anti-HA antibody



C. Co-immunoprecipitation of HA-tagged PTPσ with anti-Myc antibody

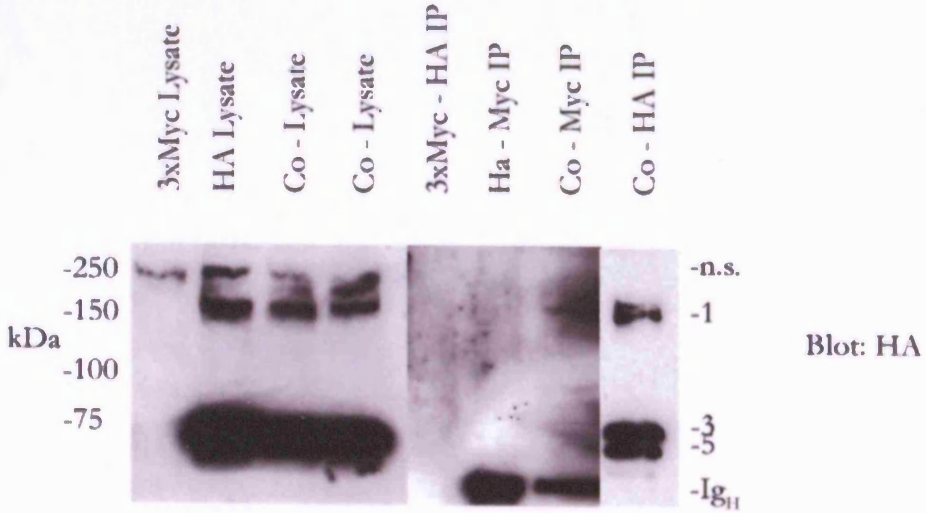


Figure 5.1: Co-immunoprecipitation of dimeric PTPσ using recombinant proteins bearing distinct intracellular epitope tags

Cell lysates and immunoprecipitates were analysed by reducing SDS-PAGE. Proteins transferred to a membrane were probed with antibodies specific for the carboxy-terminal Myc (B) or HA tags (C).

A. Schematic diagram of PTPσ proteins to show tag locations and expected cleavage products.
B. Anti-Myc detection of lysates and immunoprecipitates
C. Anti-HA detection of lysates and immunoprecipitates

Lanes:

HA Lysate: HA-expressing lysate
3xMyc lysate: Myc-expressing lysate
Co-Lysate: Lysate from co-transfected cells
3xMyc – HA IP: Anti-HA IP of 3xMyc transfected lysate
Co – Myc IP: Anti-Myc IP of co-transfected lysate

Bands:

Band 1. Full-length PTPσ 1(FL_{166})
Band 3. Phosphatase domain from amino-terminal cleavage (P_{85})
Band 5. Phosphatase domain from carboxy-terminal cleavage (P_{75})
Ig_H Reduced IgG heavy chain (Approx 50kDa)
n.s Non-specific band detected by anti-HA antibody

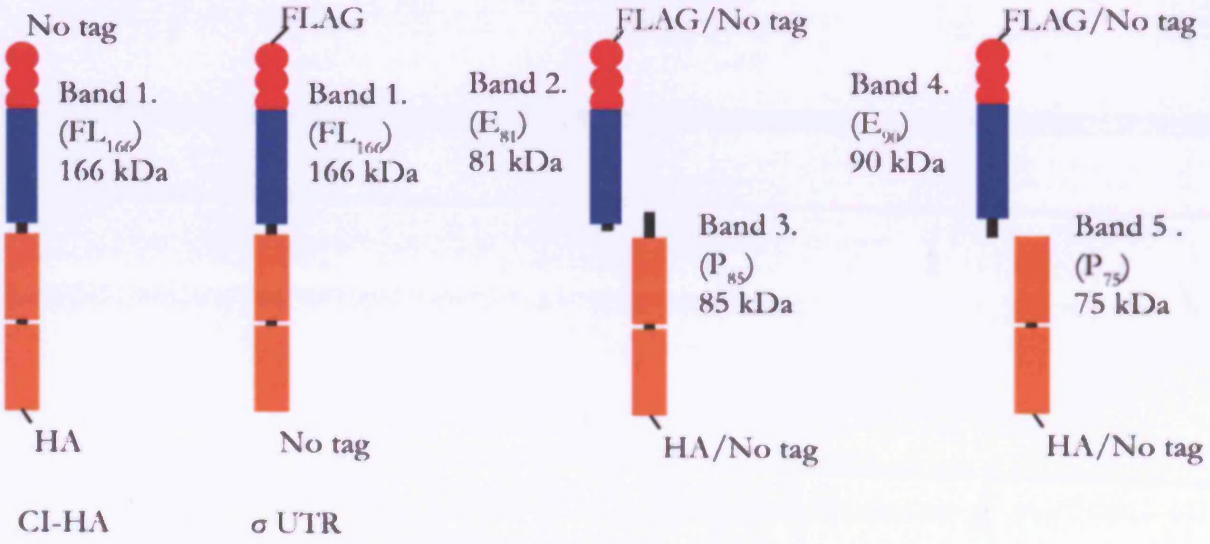
5.2: Co-immunoprecipitation using distinct intracellular and extracellular tags

As shown above, first by disulphide crosslinking and then using co-immunoprecipitation, PTPσ forms oligomeric complexes. Either a range of complexes are found some of which contain homooligomeric full-length and others, which contain homo-oligomeric $P_{85/75}$ or PTPσ forms hetero-oligomeric complexes that contain a mixture of full-length and $P_{85/75}$ PTPσ. However, the co-immunoprecipitation technique outlined above does not address whether the oligomeric complexes also contain the extracellular cleavage products. The disulphide crosslinking experiments described previously predict that the PTPσ extracellular cleavage fragments will also dimerise.

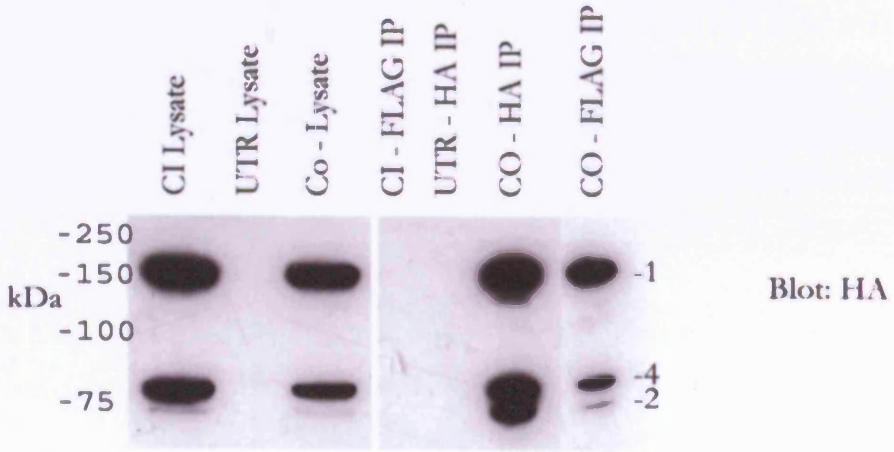
To identify whether the PTPσ oligomers also contain the extracellular cleavage fragments, two further PTPσ constructs were generated. One construct had a unique amino-terminal 3xFLAG tag (pCMV 25 PTPσ UTR) and the other a unique carboxy terminal HA tag (pCI PTPσ HA) (Figure 5.2A). There were no detectable differences with respect to cleavage or localisation when compared to other wild type PTPσ proteins (Data not shown). In addition, both proteins were expressed to high levels in HEK-293T cells and the levels of PTPσ protein were not significantly different in double- or single-transfected cells(Figure 5.2 B, C – lanes 1-4).

When lysates from cells expressing one of the differentially tagged proteins were immunoprecipitated with an antibody directed against the corresponding tag, abundant PTPσ protein was recovered (Figure 5.2B – lane 6, C – lane 7). All detectable forms of PTPσ were recovered, that is FL_{166} (band 1), E_{90} (band 2) and E_{81} (band 4). On the other hand, when lysates from cells expressing only one of the differentially tagged PTPσ proteins were immunoprecipitated with an antibody directed against the converse tag, no PTPσ protein was recovered (Figure 5.2B, C – lanes 4, 5). Moreover, when immunoprecipitates from cells expressing both lysates were immunoprecipitated with one antibody, this co-immunoprecipitated PTPσ protein labelled with the converse tag. Careful examination reveals that all three forms of PTPσ, FL_{166} (band 1), E_{90} (band 4) and E_{81} (band 2), P_{85} (band 3) and P_{75} (band 5) coimmunoprecipitate in this way (Figure 5.2B – lane 7, C – lane 6).

A. Schematic diagram of tagged constructs used in this figure



B. Co-immunoprecipitation of HA-tagged PTPσ with anti-FLAG antibody



C. Co-immunoprecipitation of FLAG-tagged PTPσ with anti-HA antibody

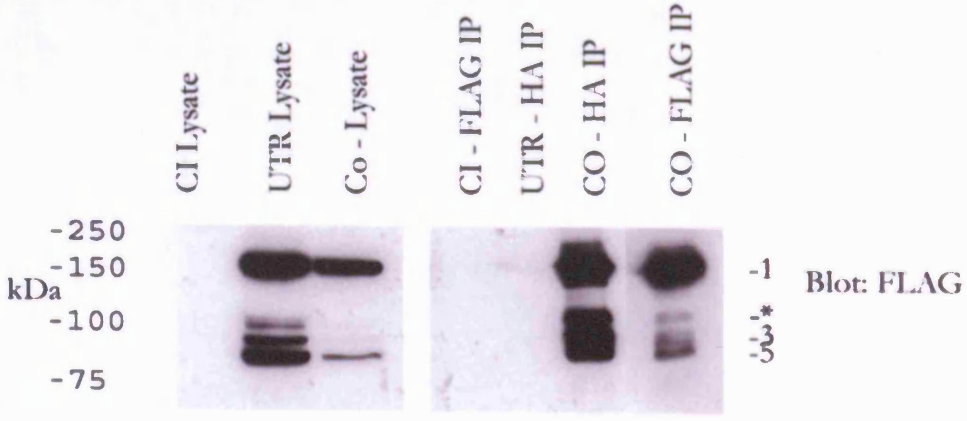


Figure 5.2 Co-immunoprecipitation of dimeric PTPσ using distinct intracellular and extracellular tags

Protein lysates and immunoprecipitates were analysed by reducing SDS-PAGE. Proteins transferred to a membrane were probed with antibodies against the amino-terminal FLAG (B) or carboxy-terminal HA tags (C).

A. Schematic diagram of PTPσ proteins to show tag locations and expected cleavage products.
B. Anti-FLAG detection of lysates and immunoprecipitates
C. Anti-HA detection of lysates and immunoprecipitates

Lanes:

CI Lysate: CI HA expressing lysate
UTR lysate: σ UTR expressing lysate
Co-Lysate: Lysate from co-transfected cells
UTR – HA IP: Anti-HA IP of σ UTR transfected lysate
Co – FLAG IP: Anti-FLAG IP of co-transfected lysate

Bands

Band 1. Full-length PTPσ 1(FL_{166})
Band 2. Extracellular domain from amino terminal cleavage (E_{81})
Band 3. Phosphatase domain from amino-terminal cleavage (P_{85})
Band 4. Extracellular domain from carboxy-terminal cleavage (E_{90})
Band 5. Phosphatase domain from carboxy-terminal cleavage (P_{75})
* Additional PTPσ cleavage product

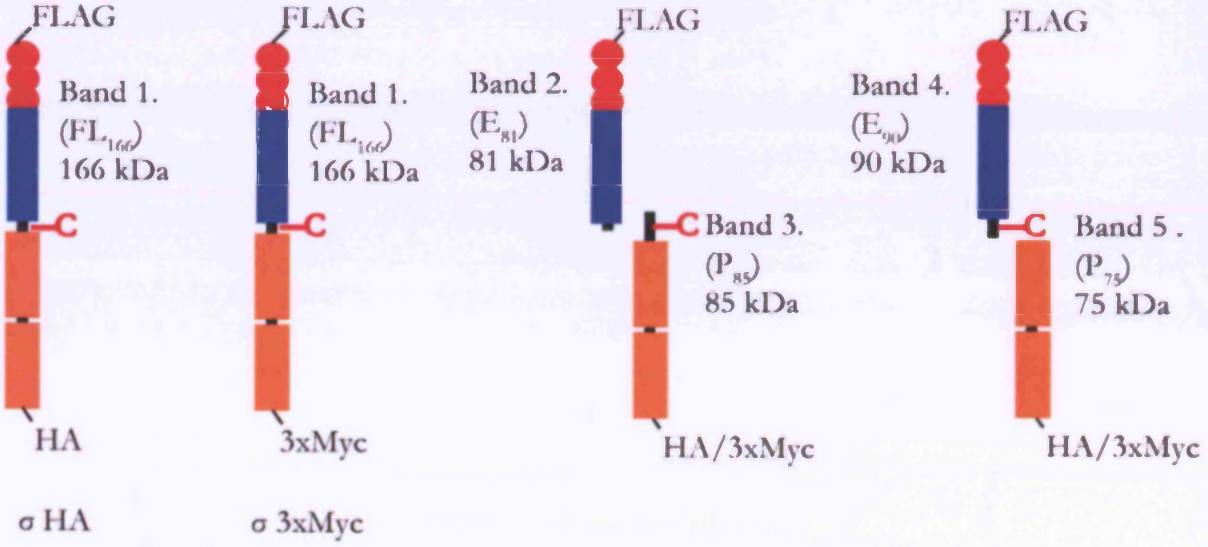
5.3: Co-immunoprecipitation of disulphide cross-linked PTPσ proteins

The identification of reduction sensitive high molecular weight species that contain specific PTPσ proteins, and the co-immunoprecipitation of differentially tagged PTPσ suggest that PTPσ forms oligomeric complexes in human cells. However, it is still possible that the reduction sensitive species represent either homooligomeric PTPσ or the interaction of PTPσ with a range of different proteins. Furthermore, even if it is granted that the disulphide crosslinking data and the co-immunoprecipitation data support a model where PTPσ forms oligomers, the exact nature of the PTPσ oligomers remains unclear. Firstly, the valency of the oligomers is unclear. Secondly, it is not known whether the same oligomeric form contains both the full-length PTPσ protein and the cleavage fragments or whether oligomers contain only full-length or cleaved protein but never both. Similarly, it is unclear whether the detection of both oligomeric intracellular cleavage products and oligomeric extracellular cleavage products reflects the existence of distinct protein complexes or a single complex consisting of oligomers of the reasssociated E_x/P_y complex.

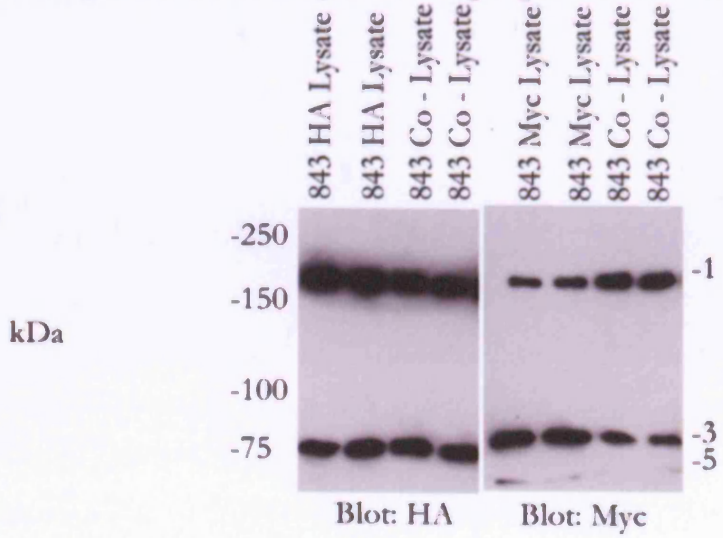
These questions can be addressed by combining the disulphide cross-linking experiments with the co-immunoprecipitation experiments. Non-reducing and two dimensional analysis of co-immunoprecipitates from cells expressing differentially tagged PTPσ would show the exact identity of the constituent parts of each high molecular weight species. Together with this information, molecular weight and quantitative analysis should accurately identify the valency of each PTPσ complex and also confirm or disprove the existence of full-length/cleaved hetero-oligomeric complexes.

Two constructs were designed such that both contained the P843C substitution and each had a different carboxy-terminal epitope tag; either an HA tag or a 3xMyc tag (Figure 5.3A). There was no detectable difference in either the localisation or cleavage pattern of these two forms of PTPσ when compared to the other constructs used in this thesis (Data not shown). Cotransfection of HEK-293T cells with these two constructs resulted in the robust expression of the two encoded proteins. Approximately equal levels of each protein were seen in lysates from single- and double-transfected cells (Figure 5.3B).

A. Schematic diagram of tagged constructs used in this figure



B. Reducing SDS-PAGE analysis showing expression of P843C PTPσ



C. Non-reducing SDS-PAGE analysis showing coimmunoprecipitation of dimeric P843C disulphide crosslinking mutant PTPσ

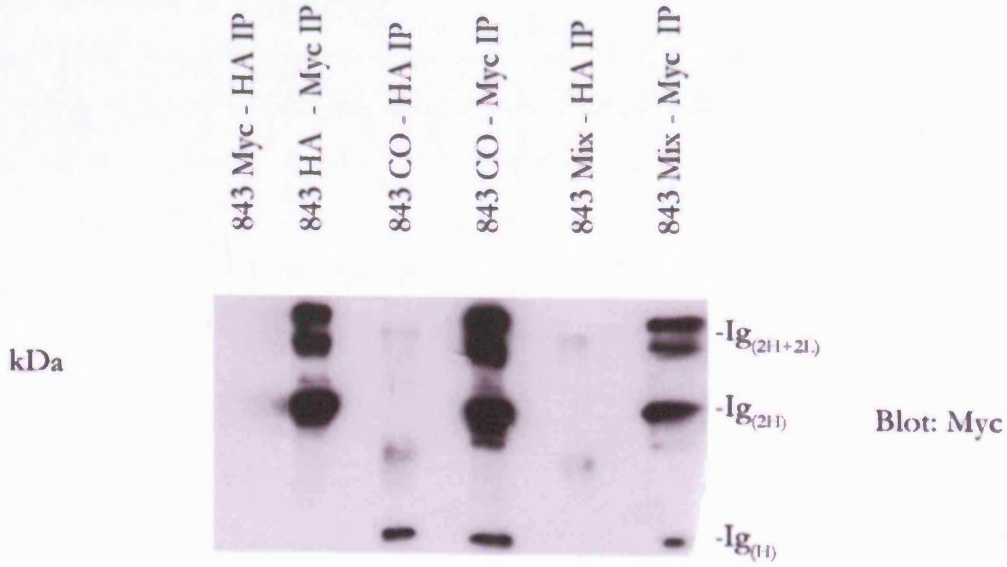


Figure 5.3: Co-immunoprecipitation of P843C disulphide cross-linked PTPσ

Protein lysates and immunoprecipitates were analysed by reducing SDS-PAGE. Proteins transferred to a membrane were probed with antibodies against the carboxy-terminal Myc (B) or HA tag (C).

A. Schematic diagram of PTPσ proteins to show tag locations and expected cleavage products.
B. Anti-HA and anti Myc detection of reducing SDS-PAGE analysis of lysates
C. Anti-FLAG detection of lysates and immunoprecipitates
D. Anti-Myc detection of non-reducing SDS-PAGE analysis of immunoprecipitates

Lanes:

843 HA lysate: PTPσ-P843C-HA expressing lysate
843 Myc lysate: PTPσ-843C-3xMyc expressing lysate
843 Co-Lysate: Lysate from co-transfected cells
843 Myc – HA IP: Anti-HA IP of PTPσ-P843C-3xMyc transfected lysate
843 Co – Myc IP: Anti-Myc IP of co-transfected lysate
843 Mix – HA IP: Anti-HA IP of mixed lysate (5.4)

Bands:

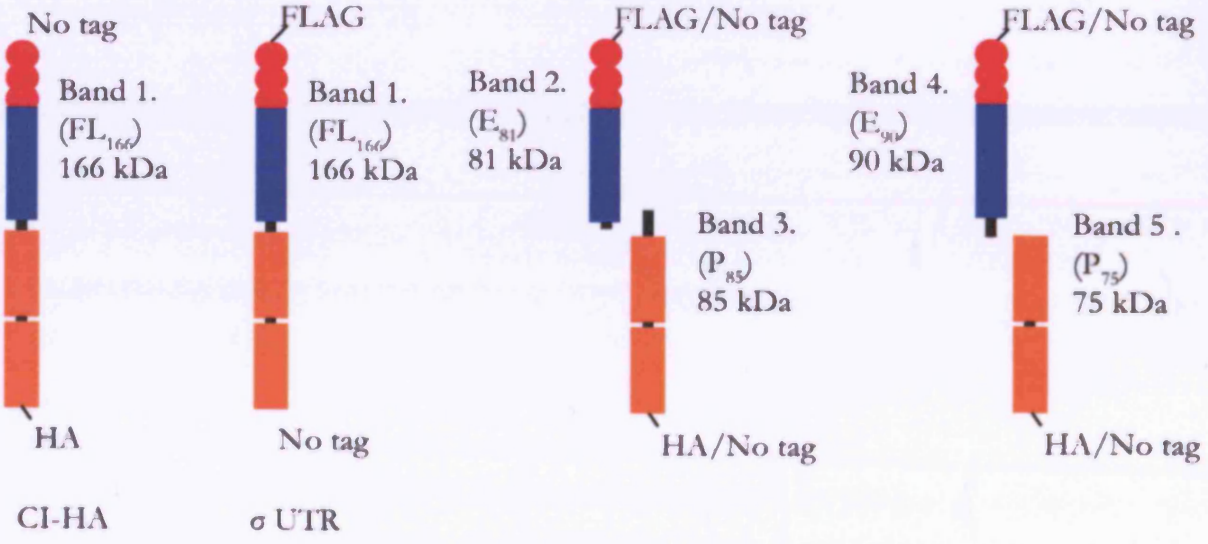
Band 1. Full-length PTPσ 1 (FL_{166})
Band 3. Phosphatase domain from amino-terminal cleavage (P_{85})
Band 5. Phosphatase domain from carboxy-terminal cleavage (P_{75})
$Ig_{(2H+2L)}$ Complete quaternary IgG molecule (Mw~160kDa)
Ig_{2H} Non-reduced divalent IgG heavy chains (Mw~100kDa)
Ig_{H} Reduced IgG heavy chain (Mw~50kDa)

Coimmunoprecipitation experiments with these constructs produced the same results as are seen with wild type PTPσ and no significant increase in the quantitative level of co-immunoprecipitating PTPσ could be detected (Data not shown). Unfortunately, it was not possible to determine whether disulphide crosslinked high molecular weight PTPσ species containing both HA and 3xMyc-tagged protein also co-immunoprecipitated in these experiments. Under non-reducing conditions, a large number of very prominent antibody bands were present at a range of sizes. Several bands are seen which presumably represent the intact IgG molecule (IgG_{2H+2L} Mw~150kDa) and several products resulting from the dissociation of this quaternary protein complex (Figure 5.3C). Unfortunately, the antibody bands were similar in size to some of the reduction sensitive PTPσ high molecular weight bands. In addition, the non-reduced antibodies generated smears that further masked the relevant regions of the gel. All attempts to clear antibody fragments from the immunoprecipitates using protein G or anti-Mouse immunoglobulins failed (Data not shown). This problem might be overcome in the future by using a solid matrix-linked affinity system that would allow the antibody bands to be efficiently separated from the proteins of interest.

5.4: Contribution of post-lysis interactions to co-immunoprecipitation

Data shown here and in chapter 4 suggest that PTPσ forms oligomeric complexes. However, these results may reflect an intrinsic tendency of PTPσ to aggregate following detergent lysis. The aggregation profile of several transmembrane and membrane-associated proteins is affected by detergent lysis (Ludi and Hasselbach, 1984; Hsu and Youle, 1998; Musatov et al., 2000). One way of examining this possibility is to mix lysates from cells each expressing a differentially tagged form of PTPσ and to compare the amount of PTPσ co-immunoprecipitated from the mixed lysates and co-expressing lysates. PTPσ protein could be co-immunoprecipitated from mixed lysates. However, the recovery was significantly lower than that achieved from lysates that co-expressed both proteins (Figure 5.4B).

A. Schematic diagram of tagged constructs used in this figure



B. Co-immunoprecipitation of FLAG-tagged PTPσ with anti-HA and anti-Myc antibodies

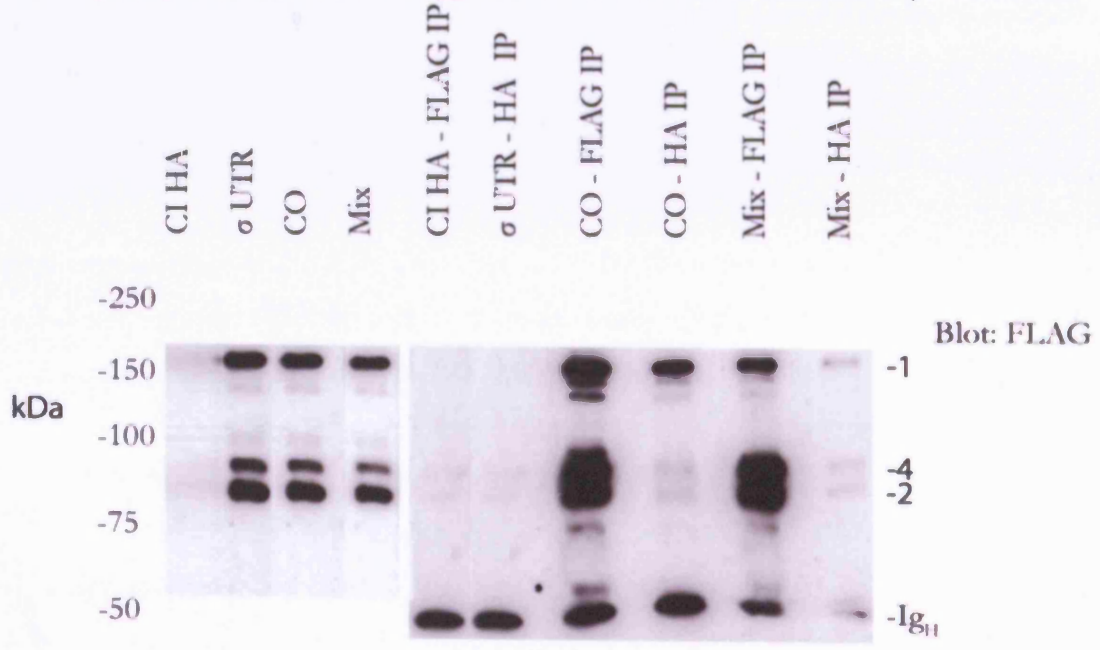


Figure 5.4: Contribution of post-lysis interactions to co-immunoprecipitation

Protein lysates and immunoprecipitates were analysed by reducing SDS-PAGE. Proteins transferred to a membrane were probed with antibodies against the amino-terminal FLAG tag.

A. Schematic diagram of PTPσ proteins to show tag locations and expected cleavage products.
B. Anti-FLAG detection of lysates and immunoprecipitates

Lanes:

CI HA: CI HA expressing lysate
σ UTR: σ UTR expressing lysate
CO: Lysate from co-transfected cells
Mix: Combined lysates from CI HA and σ UTR expressing cells
σ UTR – HA IP: Anti-HA IP of σ UTR transfected lysate
CO – FLAG IP: Anti-FLAG IP of co-transfected lysate
Mix – HA IP: Anti-HA IP of combined lysates from CI HA and σ UTR expressing cells

Bands

Band 1. Full-length PTPσ 1 (FL_{166})
Band 2. Extracellular domain from amino terminal cleavage (E_{81})
Band 4. Extracellular domain from carboxy-terminal cleavage (E_{90})
Ig_H Reduced IgG heavy chain (Approx 50kDa)

5.5: Discussion

This chapter confirms that wild type PTPσ forms oligomeric complexes as shown by co-immunoprecipitation. Prior to this work, it had only been possible to detect these interactions following biochemical crosslinking, using mutated or chimaeric proteins including disulphide crosslinking mutants, after treatment of RPTP-expressing cells with biochemical reagents or indirectly, using energy transfer techniques (Takeda et al., 1992; Desai et al., 1993; Jiang et al., 1999; Jiang et al., 2000; Tertoolen et al., 2001; Xu and Weiss, 2002; Blanchetot et al., 2002a). In the course of the work described in this thesis, dimeric RPTPs were reported using similar approaches to those described here (Gross et al., 2002; Toledano-Katchalski et al., 2003; Takeda et al., 2004; Walchli et al., 2005). In this chapter, PTPσ is shown to oligomerise without the use of chemical treatments or changes in the native PTPσ sequence other than the addition of epitope tags. Co-immunoprecipitation experiments identified oligomeric forms containing all five known PTPσ peptides (FL_{166}, $P_{85/75}$ and $E_{85/75}$). Thus, the results confirm the data seen with disulphide crosslinking mutants and extend it to include those species lacking the ectopic cysteine required for disulphide crosslinking. In addition, the demonstration of oligomerisation with co-immunoprecipitation of native sequence PTPσ confirms that the ectopic disulphide bonds do not themselves cause PTPσ dimerisation. Unfortunately, technical difficulties hindered the co-immunoprecipitation of the disulphide crosslinking PTPσ mutants (Chapter 4). In the future, combined co-immunoprecipitation/disulphide crosslinking experiments would clarify the valency and content of the PTPσ complexes.

Co-immunoprecipitation experiments with the HA- and 3xMyc-tagged PTPσ proteins show that PTPσ forms oligomeric structures that include the full-length protein and both intracellular domain cleavage products (Figure 5.1B, C). This confirms the results seen with disulphide crosslinking and extends the finding that PTPσ oligomerises to those cleavage fragments that lack an ectopic cysteine (i.e. E_{90}/P_{85}) and so were not detectable as disulphide-linked oligomers. However, this kind of immunoprecipitation experiment failed to address whether E_{81} is found in oligomeric complexes and whether individual complexes contain both extracellular and intracellular cleavage fragments or if these are maintained in separate oligomeric complexes. These questions can be addressed by co-immunoprecipitation experiments using tags that are located on either side of the cleavage sites i.e. one at the amino- and one at the carboxy-terminal end of recombinant PTPσ proteins.

Co-immunoprecipitation experiments with PTPσ proteins labelled with tags on different sides of the cleavage sites showed that PTPσ forms oligomeric complexes (Figure 5.2B, C). All labelled forms of PTPσ were seen to co-immunoprecipitate showing that E_{81} is indeed found in PTPσ oligomers. Furthermore, as the tags were located on either side of the cleavage sites several hypotheses present themselves regarding the nature of the oligomeric complexes. The co-immunoprecipitation of the cleaved forms is highly informative. Co-immunoprecipitation of homo-oligomeric complexes containing only extracellular or only intracellular domains was not detectable using this system. Therefore mixed oligomers must exist that contain either a full-length PTPσ molecule or a re-associated E/P heterodimer in addition to the cleaved domain that is being immunoprecipitated. It is notable that full-length PTPσ also co-immunoprecipitates in this system and so homooligomers of full-length protein or hetero-oligomers of full-length and re-associated E/P heterodimers must also exist. However, so far it has not been possible to identify heterodimers of cleaved and uncleaved PTPσ (Figure 4.11). Therefore, it appears that only homologous PTPσ species can be found in oligomers. It may be noted that such selectivity is unlikely to be seen if PTPσ aggregation was artefactual. However, it will be important to formally determine whether mixed full-length/cleaved PTPσ oligomers exist as this has important implications for many aspects of the biology of PTPσ including PTPσ cleavage and oligomerisation.

Hypothetical models for PTPσ oligomerisation differ principally in the state in which PTPσ cleavage occurs – monomeric or dimeric PTPσ or both. If monomeric PTPσ is subject to regulated proteolysis then a number of oligomerisation mechanisms must exist that on the one hand permit the association of PTPσ molecules with homologous patterns of cleavage but on the other hand bar the association of PTPσ molecules with heterologous cleavage patterns. Alternatively, if cleavage occurs at the oligomeric stage in such a way that cleavage of all constituent chains is linked, this might produce a number of PTPσ complexes each containing different PTPσ species. The original oligomerisation mechanism would need to be relatively unaffected by proteolysis at the two cleavage sites. However, this hypothesis does not need to postulate mechanisms for the subunit specificity of the PTPσ complexes. If cleavage were to occur at both the monomeric and oligomeric stages this would imply the same difficulties as cleavage at the monomeric stage and require enzyme mechanisms that can act in potentially quite different environments.

As alluded to above, the nature of these mixed oligomers is unclear. Although it may be assumed that these oligomers are in fact dimers, this has not been directly

demonstrated. In addition, it is not known whether a single PTPσ complex exists that may contain any of the various PTPσ peptides or if a number of complexes exist, each of which consists of a specific combination of PTPσ peptide chains. Firstly, the mechanics of PTPσ cleavage and re-association are not well understood (Serra-Pages et al., 1994). The re-associated heterodimeric E/P PTPσ complexes might result from reassociation of a single cleaved PTPσ molecule or by the assignment of cleaved extracellular domains to cleaved intracellular domains, irrespective of their molecular origin. Furthermore, it is not known whether PTPσ is cleaved in the monomeric or oligomeric state and if oligomers are cleaved it is not known whether the constituent monomers are cleaved simultaneously or sequentially. Secondly, it is unclear whether the heterodimeric E/P PTPσ complex is stable or dynamic with the exchange of individual PTPσ E domains between PTPσ P domains and their immediate subcellular environment. Although no such exchange of cleavage fragments has been reported for any RPTP, if the association of PTPσ E domains with PTPσ P domains is not restricted to those that share a common molecular origin, then these co-immunoprecipitation experiments need not suggest the formation of oligomeric complexes at all. Lastly, PTPσ co-immunoprecipitation might not reflect a direct oligomeric relationship at all, but rather the ability of a number of PTPσ peptides to associate simultaneously with a second protein. However, in the disulphide crosslinking experiments (4.3), the residual molecular weights (after subtraction of the appropriate PTPσ molecular weight) of the two major disulphide crosslinked species were different. Furthermore, the two size differences were not directly convertible by simple multiplication. Therefore, if cleaved and full-length PTPσ are not responsible for the residual molecular weight, a model must be described that uses two different proteins, not just a different number of the same unknown protein. In summary therefore, it should be emphasised that although alternative explanations exist, it is likely that PTPσ forms homodimers.

The nature of the PTPσ heterodimeric E/P complexes and the PTPσ oligomers can be addressed by combining the disulphide-crosslinking and co-immunoprecipitation experiments. Unfortunately, technical limitations have prevented such a study at the present time (5.3). Such a study would be most informative if 2 recombinant PTPσ proteins were used with a unique tag at each end of each protein; a total of four distinct tags. In addition, the interpretation of these experiments would be simplified by the use of a system that generates consistent levels of PTPσ. Historically, it has been difficult to establish a stable cell line expressing just a single PTPσ construct. Therefore, it might be desirable to avoid the necessity of generating a stable cell line expressing two PTPσ constructs

simultaneously. This might readily be achieved using a dual-cistronic system. The establishment of a cell line that stably expresses recombinant PTPσ would still be required to explore the factors that affect PTPσ cleavage. These PTPσ overexpression systems will necessarily exhibit some differences from cells that express PTPσ endogenously. However, the ability to distinguish individual PTPσ molecules is essential in order to gain a better understanding of PTPσ cleavage and oligomerisation. The results obtained in these systems can then be tested for validity in endogenous systems.

Many questions would be answered by the combined co-immunoprecipitation and disulphide cross-linking experiments outlined above. Firstly, co-immunoprecipitation of disulphide cross-linked PTPσ will formally confirm the nature of the PTPσ high molecular weight species by demonstrating whether the high molecular weight species contain the PTPσ species predicted to participate in the formation of each species. Secondly, strong ionic detergents such as SDS dissociate the E/P heterodimers (Yu et al., 1992). Immunoprecipitation of such lysates (following appropriate dilution of the detergent) should identify whether full-length/cleaved protein heterodimers form. This can be confirmed by non-reducing/reducing 2-dimensional SDS-PAGE analysis of lysates and immunoprecipitates. The presence or absence of full-length/cleaved heterodimers is of critical importance. It was shown above that PTPσ cleaved subunits can be co-immunoprecipitated across the cleavage site. If the absence of full-length/cleaved heterodimers is confirmed then this is indirect evidence that the oligomeric E subunits and P subunits associate rather than existing independently. This would be an important finding with significant implications for PTPσ oligomerisation and also the mechanisms of PTPσ cleavage. Further questions would include localising the PTPσ cleavage events to subcellular compartments and identifying the enzymes and pathways that regulate PTPσ cleavage. Understanding these events would facilitate further research on the function of type IIa RPTPs as current work is limited by the unpredictable variability of PTPσ cleavage in any given experiment.

Another notable finding from these co-immunoprecipitation experiments is that a significant proportion of cellular PTPσ co-immunoprecipitates. This suggests that a large part of the cellular PTPσ pool is oligomeric, at least under the conditions examined in this work. It may be that the high level of multimerisation detected reflects the inherent tendency of an overexpressed transmembrane domain protein to aggregate. This has previously been reported both within the cell as an artefact of overexpression or later, as an artefact of detergent lysis (Ludi and Hasselbach, 1984; Di Fiore et al., 1987; Di Marco et al.,

1990; Maru et al., 1990; Brandt-Rauf et al., 1990; Hsu and Youle, 1998; Musatov et al., 2000). However, it has already been shown that a significant amount of the full-length form of wild type and mutant PTPσ reaches the cell surface (3.2, 4.3.3) whereas non-specific membrane protein aggregation is density dependent, occurring most commonly in the organelles of the secretory pathway. Moreover, although membrane protein overexpression can increase receptor dimerisation, this usually represents an increase above physiological basal levels rather than the formation of an interaction that does not otherwise occur. The increased local density of receptor in the plasma membrane removes a major limiting influence on dimer formation. As a non-specific mechanism, it is also unlikely that aggregation would result in the formation of distinct oligomers involving only full-length or cleaved protein but not both; such a pattern is seen with the disulphide crosslinking experiments (4.3).

As mentioned above, the co-immunoprecipitation experiments can be subjected to quantitative as well as qualitative analysis. Unfortunately, several features of the PTPσ expression system used in this thesis prevent a more exact quantification of the amount of PTPσ found in the monomeric and oligomeric states. This also limits a comparison of the propensity of different PTPσ peptides to oligomerise. Firstly, it was difficult to standardise both the level of PTPσ expression and the pattern of PTPσ cleavage between experiments (Chapter 3). Furthermore, both singly- and doubly-transfected cells were present in the cell populations studied and the level of co-transfection varied between experiments. Similar variabilities affected the relative level of the two PTPσ proteins in each co-transfected cell. Furthermore, technical difficulties hindered the normalisation of co-immunoprecipitation efficiency between samples. These include the effects of oligomerisation and cleavage on epitope accessibility for co-immunoprecipitation. The effects of conformation on epitope accessibility have been clearly demonstrated for the related PTPα (van der Wijk et al., 2003). Lastly, the usefulness of such a quantification is of questionable value whilst we are ignorant of the valency of the PTPσ complexes. The proportion of PTPσ that co-immunoprecipitates as part of an oligomeric complex depends both on the level of PTPσ oligomerisation and the complex valency. If the complexes are dimeric, at most only 50% of each monomer can be co-immunoprecipitated with antibodies against the other subunit. In complexes with higher valencies, this limit rapidly approaches 100% (e.g. Trimer – 75%; tetramer - 87.5% etc).

Two points can be made regarding PTPσ oligomerisation. Firstly, a significant proportion of cellular PTPσ is found in oligomeric complexes. This level is similar to that

detected with disulphide crosslinking techniques. Although these are not formally compared in this work, this suggests that disulphide crosslinking reflects rather than mediates PTPσ oligomerisation. Secondly, there was some variability in the relative proportion of each co-immunoprecipitating species, both with respect to each other and in comparison with their relative abundance in the lysates (Figure 5.1B, C). However, there was no consistent trend to these differences and as described above the relative abundance of each band varied from experiment to experiment both in the lysates and in the resulting co-immunoprecipitates. As all detectable tagged species co-immunoprecipitated, the observed differences are more likely to represent interexperimental variation rather than real differences in the propensity of each species to oligomerise. Indeed, as previously discussed, the absolute PTPσ level and the PTPσ cleavage pattern varied significantly from experiment to experiment (Chapter 3).

Lastly, it is worth noting that the PTPσ oligomerisation seen is not primarily mediated by interactions that occur after cell lysis (Figure 5.4). Such interactions have been described for other transmembrane receptors following detergent solubilisation. Some PTPσ oligomerisation is detected following the combination and subsequent immunoprecipitation of lysates from cells each expressing only a single PTPσ construct. However, the level of association detected in this way is significantly lower than that which is detected in lysates from cells co-expressing two differentially-tagged PTPσ proteins.

In fact, the low level of oligomerisation detected in combined lysates need not represent artefactual PTPσ aggregation at all. PTPσ molecules may retain the ability to interact in solution when solubilised with the mild non-ionic detergents used in this study. This interaction may reflect a *cis*- or *trans*-dimerisation mechanism similar to that seen in the related type IIb RPTPs or binding of PTPσ to a divalent interacting protein present in the lysate. Such possible interactions include reverse signaling mechanisms where PTPσ acts as a ligand rather than a receptor. Previously well characterised for Ephrins, this type of interaction has recently been suggested for PTPσ extracellular domains although the receptor involved has not been identified (Holland et al., 1996; Mellitzer et al., 2000; Sajnani et al., 2005). Finally, the size of detergent micelles and the rate of exchange of solubilised PTPσ molecules between micelles is unknown.

The experiments reported in this chapter support the data presented in chapter 4 and suggest that PTPσ is largely dimeric. This is in agreement with the situation seen with type IV and type IIb RPTPs. The data presented here extend the disulphide cross-linking

data to show that the PTPσ species that lack the ectopic cysteine required for disulphide crosslinking also form dimers. Therefore, all known PTPσ peptides, both cleaved and full-length form oligomers. A hypothetical model for PTPσ oligomerisation and cleavage was presented. Briefly, this model proposes that PTPσ is highly oligomeric and that it is the oligomers that are subject to cleavage. Cleavage on all the chains in an oligomer is coupled such that hetero-oligomers of full-length and cleaved PTPσ are undetectable. An experimental system that might resolve some of the questions that remain in this area was presented and discussed.

Chapter 6: Effects of chemical mediators on PTPσ

This chapter extends the descriptive work carried out in Chapter 3, confirming previous work, which showed that PTPσ cleavage and shedding may be induced by protein kinase C (PKC) activation (Aicher et al., 1997). However, this chapter also shows that in addition to the shed E_{81} fragment, the E_{90} fragment is shed in a dose-responsive manner following PKC activation by a phorbol ester. In addition, the effects of treatment with the tyrosine phosphatase inhibitors, sodium pervanadate and H_2O_2, are reported. Pervanadate treatment and hence tyrosine phosphatase inhibition has no effect on PTPσ expression level, cleavage pattern or high molecular weight species formation. However, a cleavage fragment shows preferential tyrosine phosphorylation at an unknown site, which suggests an as-yet unclear interaction between PTPσ phosphorylation and at least one of the two proteolytic cleavage events. In contrast, it is shown that H_2O_2 induces the formation of high molecular weight forms of PTPσ. This suggests that H_2O_2 has effects on RPTP expressing cells that are distinct from pure tyrosine phosphatase inhibition. This is in agreement with reports of the formation of high molecular weight species by PTPα and an PTPα-LAR chimera in cells treated with H_2O_2 (van der Wijk et al., 2004). As H_2O_2 is produced in response to growth factor signaling, the effect of serum on high molecular weight species formation was evaluated but no effect was seen on PTPσ expression level, cleavage pattern or high molecular weight species formation. Lastly, the effect of heparin treatment of PTPσ-transfected cells was evaluated. A number of Heparan sulphate proteoglycans (including heparin) are known to interact with PTPσ (Aricescu et al., 2002). However, heparin treatment has no discernable effect on PTPσ expression level, cleavage pattern or high molecular weight species formation.

6.1: Treatment with the phorbol ester, TPA

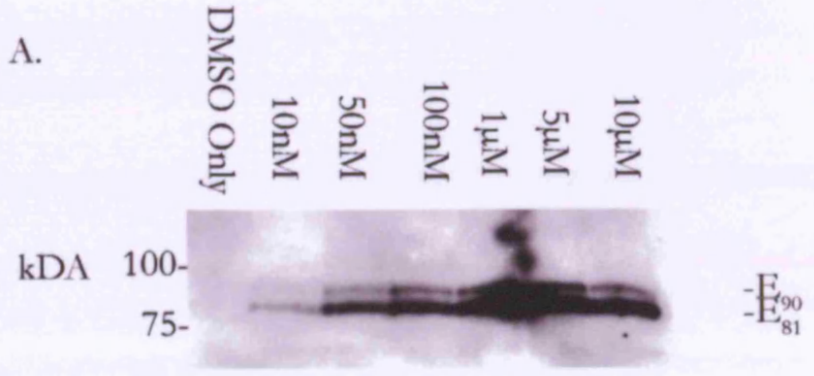
Among the most prominent features of PTPσ processing are the proteolytic cleavage events. The effect of these events on PTPσ function is not understood and so it will be important to identify the factors regulating the two PTPσ cleavage events and the effects of each on PTPσ function. The carboxy-terminal (secondary) cleavage event can be stimulated by Protein Kinase C (PKC) activation or calcium ionophore treatment, resulting in intracellular domain internalisation and soluble extracellular domain shedding (Aicher et al., 1997). Similar processes are a feature of a number of membrane proteins (Jung and Dailey, 1990; Pandiella and Massague, 1991; Gandy and Greengard, 1994). However, type IIa RPTPs are different from many of these proteins in that they have two cleavage sites

allowing the production of E_{90}/P_{75} fragments in addition to E_{81}/P_{85} fragments (Serra-Pages et al., 1994; Aicher et al., 1997). The significance of the additional amino-terminal cleavage event is not clear. The current model suggests that PTPσ is constitutively cleaved by an subtilisin-type protein convertase at its amino-terminal site during or immediately after maturation in the endoplasmic reticulum and Golgi network (Streuli et al., 1992; Jiang et al., 1993; Yan et al., 1993; Serra-Pages et al., 1994; Pulido et al., 1995a; Campan et al., 1996; Cheng et al., 1997). Subsequently, inducible carboxy-terminal cleavage, which is mediated by a metalloprotease, is thought to occur at a membrane proximal site and release soluble extracellular domain from the reassociated E_{81}-P_{85} heterodimer (Serra-Pages et al., 1994; Aicher et al., 1997; Ruhe et al., 2006; Anders et al., 2006). However, as shown above, significant amounts of the E_{90} species can be identified in cell lysates and it does not generally accumulate in the media in the same way as the E_{81} fragment (Chapter 3, 4). Furthermore, although a formal description of this larger E form is lacking in many papers, it has previously been identified in SDS-PAGE gels of sample from both over- and endogenously-expressing type II RPTP systems (Serra-Pages et al., 1994; Pulido et al., 1995a; Aicher et al., 1997; Suarez et al., 1999; Ostenson et al., 2002). This implies that carboxy-terminal cleavage alone is not sufficient for PTPσ shedding. Therefore, HEK-293T cells transfected with wild-type PTPσ were treated with a PKC activator to determine whether the increase in carboxy-terminal cleavage previously reported to result in an increase in the soluble E_{81} fragment also results in an increase in the amount of the cell associated or soluble E_{90}.

HEK-293T cells transfected with wild type PTPσ release significant amounts of the E_S fragment into the tissue culture media over time (3.1). Following the replacement of their media, treatment of these cells with phorbol ester (Phorbol-12-myristate-13-acetate (TPA)) for five minutes resulted in the rapid appearance of PTPσ cleavage fragments in the media (Figure 6.1). In independent experiments, a dose-responsive increase in the amount of shed E_{81} was seen. The effect of TPA reached a plateau in the region of 1-5μM but was detectable at 10nM. It is of note that E_{90} is also detected in the media of TPA-treated cells. The amount of shed E_{90} also increases in proportion to the TPA concentration and the amount of shed protein again peaks at treatment levels around 1-5μM. The amount of shed E_{90} is significantly less than the amount of E_{81}. Following harvesting, media was passed through a 0.22μm filter and no full-length PTPσ was detected in the media. In addition, there was no significant difference in the amount of cellular PTPσ that paralleled the difference seen in the shed protein. Therefore, it is likely that the E_{90} seen represents shed

protein and not artefactual cell-associated protein that has passed through the filter into the supernatant. As TPA-induced PTPσ cleavage has previously been reported, PKC activation was not formally confirmed (Aicher et al., 1997). However, it would be possible to confirm PKC activation by showing an increase in the phosphorylation state of known PKC targets using phosphospecific antibodies. It was not possible to determine whether TPA treatment had an effect on PTPσ dimerisation or high molecular weight species formation due to the spatial relationship between the cleavage sites and the disulphide cross-linking sites. In addition, the TPA vehicle (Dimethylsulphoxide) has a marked effect on the amount of immunoprecipitatable PTPσ, possibly through structural effects on the epitope tags (Data not shown). In summary therefore, the effect of cleavage and shedding on dimerisation and indeed the effect of dimerisation on cleavage and shedding are important unanswered questions that need addressing.

A.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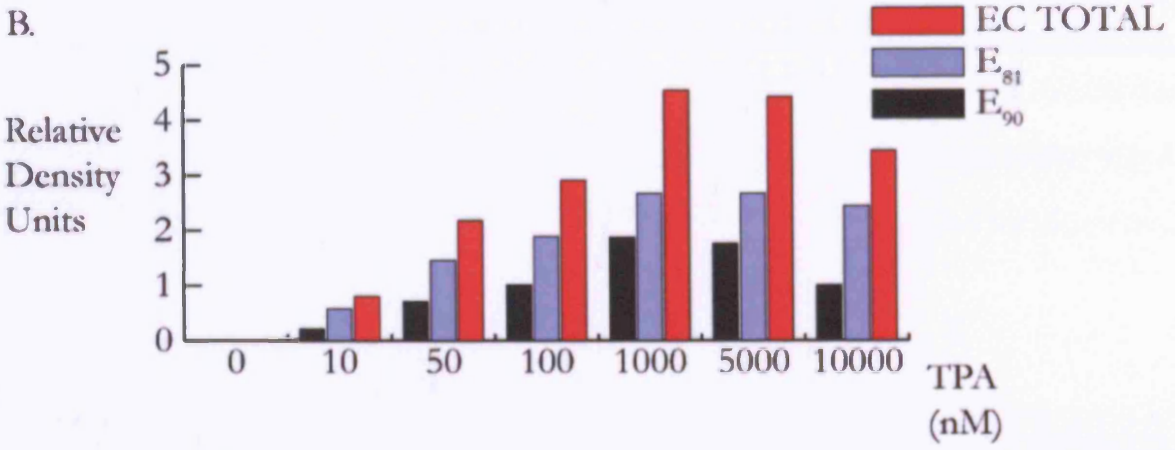
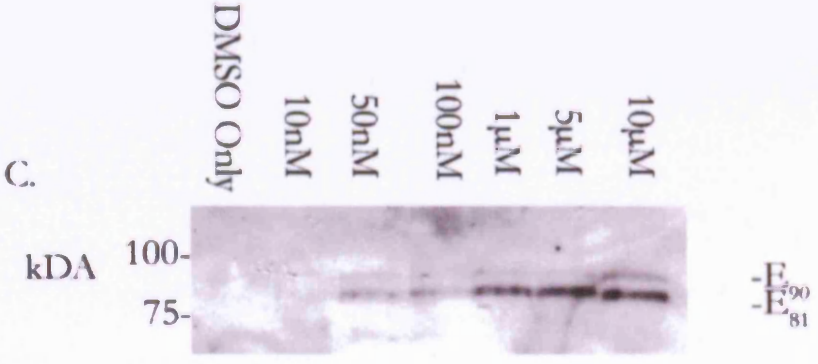
50nM
100nM
1μM
5μM
10μM
kDA
100-
75-
-E90
-E81

B.


EC TOTAL
E81
E90
Relative Density Units
5
4
3
2
1
0
0
10
50
100
1000
5000
10000
TPA (nM)

C.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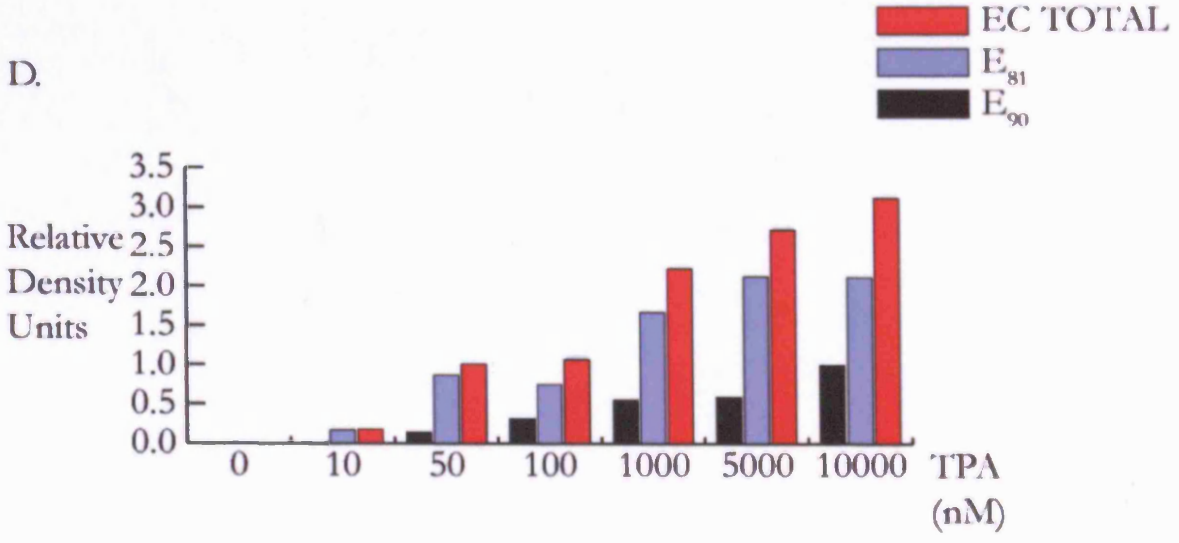
50nM
100nM
1μM
5μM
10μM
kDA
100-
75-
-E90
-E81

D.


EC TOTAL
E81
E90
Relative Density Units
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3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
50
100
1000
5000
10000
TPA (nM)

Figure 6.1: Treatment of PTPσ-transfected HEK-293T cells with Phorbol-12-myristate-13-acetate (TPA) causes PTPσ extracellular domain shedding

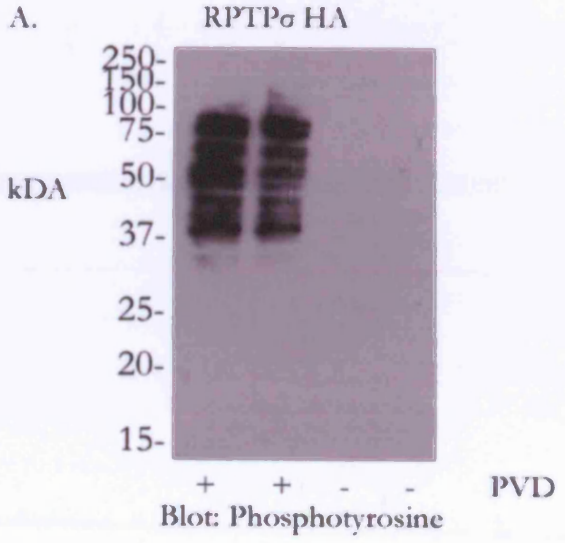
After media replacement, FLAG-tagged PTPσ transfected HEK-293T were treated for one hour with a range of concentrations of phorbol ester (Phorbol-12-myristate-13-acetate (TPA)) to induce protein kinase C activation. The supernatant was harvested and the cells were lysed. The media was passed through a 0.22μm filter to remove cells and debris and analysed by SDS-PAGE. PTPσ extracellular domain fragments present in the media were detected using the FLAG (Amino-terminal) tag. PTPσ fragments (E_{90}/E_{81}) are marked to the right of the figure. The autoradiographs were scanned using a BioRad Densitometer and the amount of the E_{90} and E_{81} species determined separately by densitometry (NIH Image). The separate values were also combined to give a total amount of shed extracellular domain in each sample.

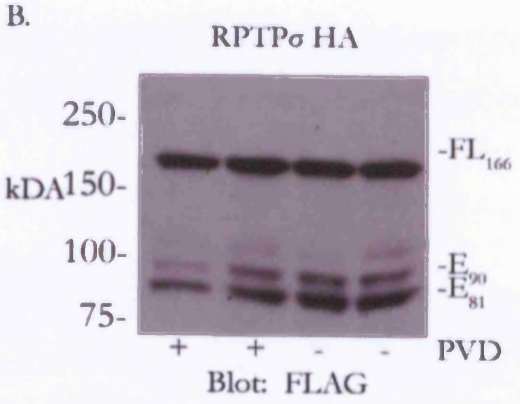
A. SDS-PAGE analysis of media from one TPA treatment experiment. TPA was added to the concentration indicated except in the DMSO only experiments where the amount of DMSO added was equal to the highest amount of vehicle used as the TPA vehicle (100nM/10μM – $1\%_{(v/v)}$).
B. Densitometric analysis SDS-PAGE gel A.
C. SDS-PAGE analysis of media from second, independent TPA treatment experiment.
D. Densitometric analysis of SDS PAGE gel C.

6.2: Treatment with pervanadate

Activation of the serine/threonine kinase, Protein kinase C (PKC), induces PTPσ cleavage. In addition to their regulation by serine/threonine kinases, preliminary evidence exists to suggest that RPTPs have the ability to autodephosphorylate and so are probably also regulated by tyrosine phosphorylation (Aricescu, 2002). Little is known about the effects of tyrosine phosphorylation on RPTPs. Several receptor protein families undergo proteolytic cleavage in response to tyrosine phosphatase inhibition (Vecchi et al., 1998; Codony-Servat et al., 1999). It is not known whether tyrosine phosphorylation affects PTPσ expression level, cleavage or high molecular weight formation. PTPσ-transfected cells were treated with the powerful tyrosine phosphatase inhibitor pervanadate to determine if PTPσ is phosphorylated *in vivo* and whether this has any effect on PTPσ expression level or cleavage pattern. Although nominally a cysteine-based tyrosine phosphatase inhibitor, by preventing the dephosphorylation of the tyrosine kinase activation loop tyrosine pervanadate also stimulates tyrosine kinase activity, resulting in a raised cellular phosphotyrosine level.

Pervanadate treatment of PTPσ-transfected HEK-293T cells reproducibly and significantly increased the overall phosphotyrosine level of the cell lysates (Figure 6.2A). However, despite the marked effect on phosphotyrosine levels, PTPσ cleavage and expression level were unaffected with respect to both the amino- and carboxy-terminal cleavage fragments (Figure 6.2B, C). Analysis of PTPσ immunoprecipitates from these cell lysates revealed that both full-length PTPσ and its cleaved intracellular domain fragments (P_{85}, P_{75}) became phosphorylated following PVD treatment (Figure 6.2D). However, the cleaved intracellular domain was significantly more phosphorylated than the full-length molecule despite significantly more full-length protein being present in the lysates and approximately similar amounts of cleaved and full-length protein immunoprecipitating (Figure 6.2C, D, E). The effect of PTP inhibition on high molecular weight species formation or dimerisation was not assessed.


A.
RPTPσ HA
250-
150-
100-
75-
kDA
50-
37-
25-
20-
15-
+ + - - PVD
Blot: Phosphotyrosine


B.
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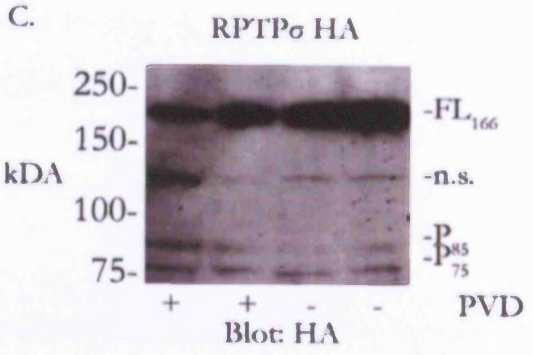
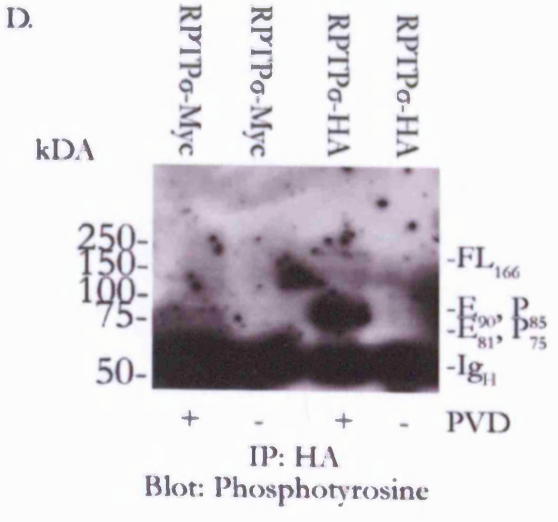
kDA150-
100-
75-
-FL166
-E90
-E81
+ + - - PVD
Blot: FLAG


C.
RPTPσ HA
250-
150-
kDA
100-
75-
-FL166
-n.s.
-P85
-P75
+ + - - PVD
Blot: HA


D.
RPTPσ-Myc
RPTPσ-Myc
RPTPσ-HA
RPTPσ-HA
kDA
250-
150-
100-
75-
50-
-FL166
-E90, P85
-E81, P75
-IgH
+ - + - PVD
IP: HA
Blot: Phosphotyrosine

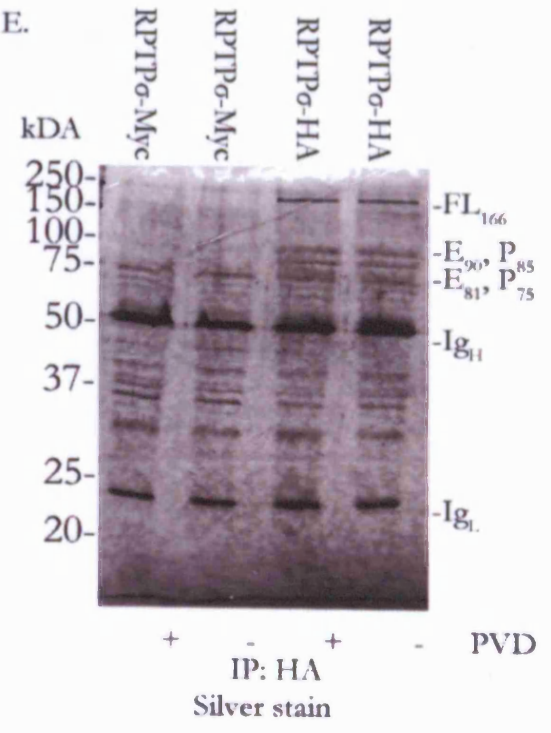

E.
RPTPσ-Myc
RPTPσ-Myc
RPTPσ-HA
RPTPσ-HA
kDA
250-
150-
100-
75-
50-
37-
25-
20-
-FL166
-E90, P85
-E81, P75
-IgH
-IgL
+ - + - PVD
IP: HA
Silver stain

Figure 6.2: Effects of sodium pervanadate on PTPσ cleavage and expression level

Cells transfected with various forms of PTPσ (FLAG-(Amino-terminal), HA/Myc-tagged (Carboxy-terminal)) were treated with the non-specific tyrosine phosphatase inhibitor, sodium pervanadate (PVD). The effects of tyrosine phosphatase inhibition on cellular phosphotyrosine, PTPσ expression and cleavage and on PTPσ phosphorylation were determined. PTPσ peptides, immunoglobulin bands (Heavy – Ig_H (50kDa), Light – Ig_L (25kDa)) and non-specific (n.s.) bands are marked to the right of the gels.

A. Phosphotyrosine (4G10) detection of total lysates from PTPσ transfected cells treated either with control (-) or 1mM PVD (+) for 15minutes.
B. Amino-terminal (FLAG) detection of total lysates from PTPσ transfected cells treated either with control (-) or 1mM PVD (+) for 15 minutes.
C. Carboxy-terminal (HA) detections of total lysates from PTPσ transfected cells treated either with control (-) or 1mM PVD (+) for 15minutes.
D. Phosphotyrosine detection (4G10) of immunoprecipitates (HA) from PTPσ-HA or PTPσ-Myc transfected cells treated with either control (-) or 100μM PVD (+) for 30 minutes.
E. Total protein detection (Neutral silver stain) of immunoprecipitates (HA) from PTPσ-HA or PTPσ-Myc transfected cells treated with either control (-) or 100μM PVD (+) for 15 minutes.

6.3: Treatment with hydrogen peroxide

A second non-specific, cysteine-based-tyrosine-phosphatase inhibitor, H_2O_2, has recently been reported to affect the conformation and possibly oligomeric state of PTPα (van der Wijk et al., 2003; van der Wijk et al., 2004). Historically, H_2O_2 was thought to act as an RPTP inhibitor through oxidation of the active site cysteine (C215S D1) in the membrane proximal phosphatase domain, which is essential for the mechanism of cysteine-based RPTPs (Huyer et al., 1997; Denu and Tanner, 1998). This data was largely extrapolated from work on the cytoplasmic PTP1B, which has only a single phosphatase domain. However, recent data shows that the oxidative effects of H_2O_2 are expressed largely by oxidation not of the membrane proximal phosphatase domain catalytic cysteine but of the active site cysteine in the carboxy-terminal tyrosine phosphatase domain, which is not thought to have significant catalytic activity (Persson et al., 2004; Groen et al., 2005). Therefore, it appears that H_2O_2 may mediate at least part of its effect by a novel mechanism in addition to oxidation of the catalytically active tyrosine phospatase domain active site cysteine. Given the data suggesting that PTPσ is inhibited by dimerisation, it is interesting to note that PTPα inhibition by H_2O_2 is associated with a possible increase in PTPα dimerisation (van der Wijk et al., 2004). This is of particular interest as H_2O_2 is a physiological cellular second messenger produced in response to growth factor stimulation and so might play a role in the functional regulation of PTPσ (Sundaresan et al., 1995; Bae et al., 1997; Meng et al., 2002). The effect of H_2O_2 on PTPα dimerisation has been shown also to apply to a chimaeric PTPα-LAR fusion protein. Therefore, the effect is either mediated purely through the PTPα extracellular domain and HA tag or H_2O_2 induced dimerisation is a feature of several PTPs. The effect of H_2O_2 on PTPσ expression level, cleavage pattern and high molecular weight species formation was determined by treating PTPσ-transfected cells with different concentrations of H_2O_2.

Initial experiments failed to detect any high molecular weight PTPσ species on treatment with H_2O_2 (Figure 6.3E). This is especially remarkable when compared to the amount of high molecular weight species formation seen in Chapter 4. However, in the experiments shown in Chapter 4, so much high molecular weight product was detected that the gels were difficult to interpret. Therefore, the experimental protocol was optimised to minimise high molecular weight smear formation, except that mediated through the ectopic cysteine residues. Significantly, during the optimisation of high molecular weight smear formation (Chapter 4), lysates containing Nonidet P-40 (NP40) but lacking iodoacetamide produced lower levels of high molecular weight smears than similar lysates including Triton

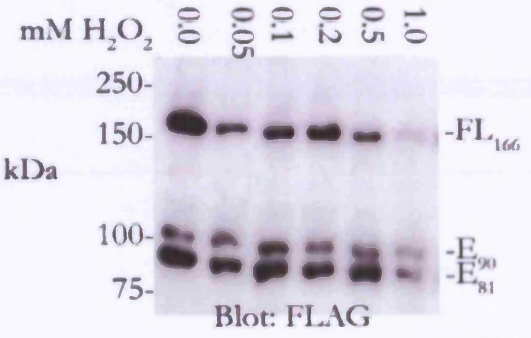
X-100 (TX100) (Data not shown but compare Figure 4.6 and Figure 6.4). Although these two detergents are similar in many ways, a noticeable difference is that many commercial batches of TX100 contain significant levels of H_2O_2 (~65mM; 1% lysis buffer = 0.65 mM) whereas NP40 (<0.06mM; 1% lysis buffer < 0.00065 mM) has a 100,000-fold lower H_2O_2 concentration (Ashani and Catravas, 1980; Roche datasheets). This hypothesis supported the PTPα data and suggested that H_2O_2 might induce PTPσ to form high molecular weight species.

The failure of the tyrosine phosphatase inhibitor, H_2O_2, to increase cellular phosphotyrosine under these conditions was unexpected. The most likely explanation was a technical error, although H_2O_2-induced phosphatase inhibition has not previously been reported to be a fastidious treatment. The most likely problem was that the way in which the H_2O_2 was presented might not be optimal. A detailed review of the available literature showed that although the optimal conditions for H_2O_2-induced tyrosine phosphatase inhibition have not been widely discussed, the conditions for H_2O_2-induced lipid peroxidation have been carefully optimised. Firstly, H_2O_2-induced lipid peroxidation requires a source of Fe^{2+} and is inhibited by common media additives including Phenol Red and tyrosine (Dugas et al., 2000). The standard growth media for HEK-293T cells, as used in these experiments is Dulbecco's modified essential media (DMEM). DMEM contains 10% $_{(v/v)}$ foetal calf serum, only an Fe^{3+} source (Fe – 0.1 mg/L $Fe(NO_3)_3$) and high concentrations of phenol red (PR – 16 mg/L) and tyrosine (Tyr – 104 mg/L) (American Tissue Culture Collection website; Sigma-Aldrich media formulations). Although replacing growth media with Dulbecco's phosphate buffered saline (DPBS) prior to stimulation removes contaminating catalyse, tyrosine and Phenol Red, DPBS lacks an Fe^{2+} source. However, HEK29T cells also grow well in other media such as Ham's nutrient mixes F10 (PR – 1.3mg/L; Tyr – 2.6mg/L; Fe – 0.8mg/L $Fe(SO_4)$) and F12 (PR – 1.3mg/L; Tyr – 7.8mg/L; Fe – 0.8mg/L $Fe(SO_4)$), which both have more suitable components for H_2O_2 treatment. In addition, a careful literature review revealed that possibly for serendipitous reasons all published work on RPTPs and H_2O_2 used either one of Ham's nutrient mixes or a media containing equal proportions of DMEM and one of the Ham's mixes (Blanchetot et al., 2002a; van der Wijk et al., 2003; van der Wijk et al., 2004; Persson et al., 2004). Peroxide treatment of PTPσ-transfected cells treated using the revised conditions induced small but detectable changes in cellular phosphotyrosine levels (Figure 6.3F).

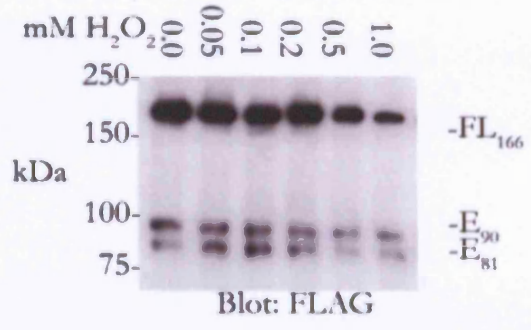
H_2O_2 does not affect the cleavage pattern of PTPσ but does decrease PTPσ protein level in a manner that requires an intact active site cysteine in the membrane distal

phosphatase domain. Lysates from H_2O_2-treated cells expressing wild type or cysteine mutant (C215S Domain 1 (cPTPσ aa: 1141), C215S Domain 2 (cPTPσ aa: 1432), C866L) were examined to determine if H_2O_2 affects the cleavage pattern of PTPσ. SDS-PAGE analysis of PTPσ-transfected cell lysates failed to show any effect of H_2O_2 on PTPσ cleavage patterns. However, in a number of duplicate experiments there was a suggestion that H_2O_2 treatment reduced the overall abundance of PTPσ proteins. The effect appeared to be least marked in cells expressing the C215SD2 mutation (Figure 6.3A-D).

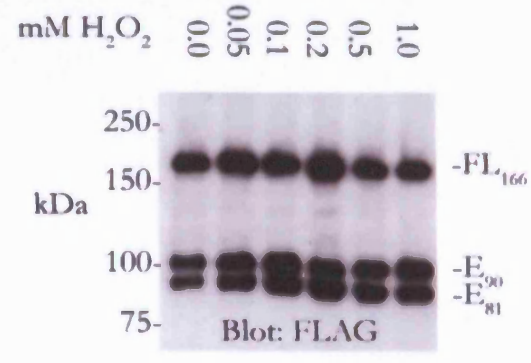
A. Wild type



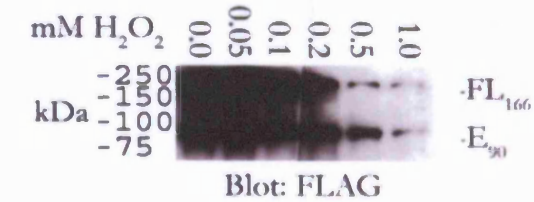
B. C215S Domain 1



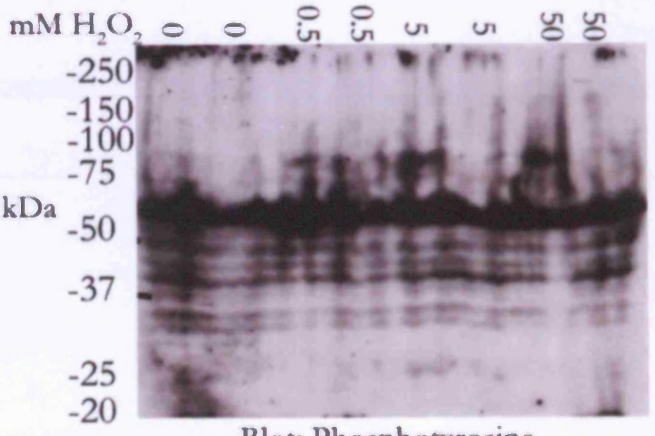
C. C215S Domain 2



D. C866L



E. Original conditions



F. Improved conditions

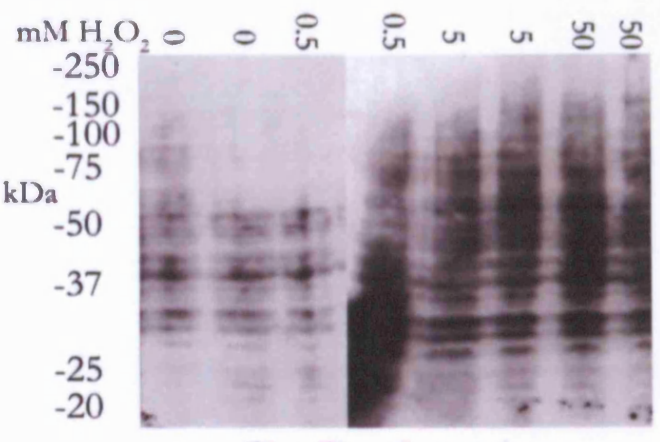


Figure 6.3: Effect of H_2O_2 treatment on PTPσ cleavage and expression level

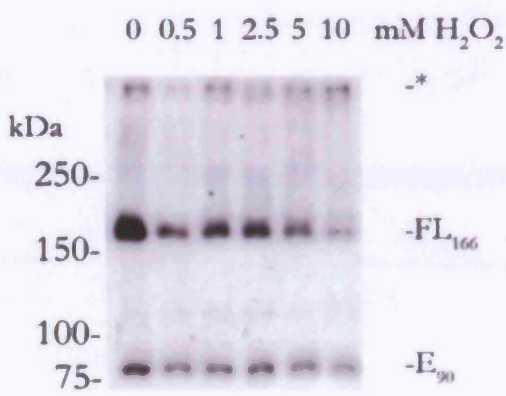
HEK-293T cells cultured in F10 media with 10% foetal calf serum were transfected with wild type or mutant PTPσ (C215S domain 1, C215S domain 2, C866L). The cells were treated with hydrogen peroxide for 5 minutes at a range of concentrations. Before treatment, the serum-containing media was replaced with serum-free media. Cells were lysed in a buffer lacking iodoacetamide. The lysates were analysed using reducing SDS-PAGE. Western analysis used an antibody directed against the FLAG (amino-terminal) tag. PTPσ peptides are marked at the right hand edge of the figure. Equal total amounts (Bradford assay) of protein were loaded in each lane.

A. Wild type PTPσ
B. C215S Domain 1 mutant PTPσ
C. C215S Domain 2 mutant PTPσ
D. C866L mutant PTPσ
E. Original H_2O_2 treatment conditions. Cells were treated for 5 minutes with the indicated concentration of H_2O_2 diluted in serum-free DMEM media. Phosphotyrosine levels were detected using an anti-phosphotyrosine antibody.
F. Improved H_2O_2 treatment conditions. Cells were treated for 5 minutes with the indicated concentration of H_2O_2 diluted in serum-free F10 media. Phosphotyrosine levels were detected using an anti-phosphotyrosine antibody.

H_2O_2 treatment causes an increase in the formation of high molecular weight species by PTPα, which depends upon endogenous cysteine residues (van der Wijk et al., 2004). In addition, it was suggested above that H_2O_2 treatment causes a decrease in the total level of PTPσ protein, which requires the catalytic cysteine in the membrane distal phosphatase domain. Having established suitable conditions for H_2O_2 treatment of PTPσ-transfected cells, it became possible to test the hypothesis that H_2O_2 stimulates the formation of high molecular weight species by PTPσ-transfected cells. HEK-293T cells transfected with wild type, C215SD1, C215SD2 and C866L PTPσ were treated with H_2O_2 at a range of concentrations. Initially, cells were lysed in buffers lacking the cysteinyl-modifying agent, iodoacetamide and PTPσ protein species were analysed by non-reducing SDS-PAGE. In the absence of iodoacetamide, wild-type PTPσ formed reduction sensitive high molecular weight smears whereas PTPσ proteins containing the C215SD1, C215SD2 or C866L mutations contained markedly less PTPσ protein in this size range (Figure 6.4A-D). H_2O_2 treatment decreased the overall signal level of PTPσ species seen on immunoblot analysis of non-reduced samples from transfected cells. Again, the effect was least marked in lysates from cells expressing the C215SD2 mutation. Therefore, the C215SD1, C215SD2 and C866L mutations appear to reduce high molecular weight smear formation under these conditions. However, in comparison to work on PTPα, it should be noted that it was necessary to use higher H_2O_2 concentrations with PTPσ and to overexpose these gels to detect PTPσ high molecular weight species. Therefore under these conditions, H_2O_2-induced high molecular weight species formation was less prominent with PTPσ than with PTPα (van der Wijk et al., 2004).

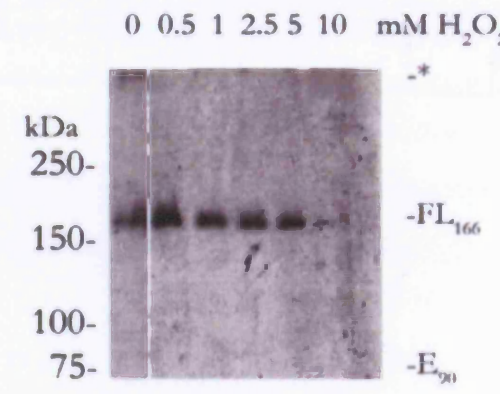
The presence of iodoacetamide (IAA) in the lysis buffer decreased high molecular weight smear formation in untreated wild type PTPσ expressing cells (Chapter 4). However, the addition of IAA into the lysis buffer for the H_2O_2 experiments had a significant and unexpected effect. In the presence of IAA, wild type and mutant (C215S D1, C215S D2 and C866L) PTPσ proteins formed significantly greater amounts of high molecular weight species, and at a lower H_2O_2 concentration, than in the absence of IAA. Moreover, discrete bands were seen rather than the smears reported previously for wild type PTPσ (Chapter 4). However, the abundance of these forms was still less than seen with PTPα. In addition, the amount of H_2O_2-induced high molecular weight band clearly increased in a dose-responsive manner and the amount of total PTPσ appeared to decrease reciprocally in both wild type and mutant PTPσ (Figure 6.5A-D).

A.



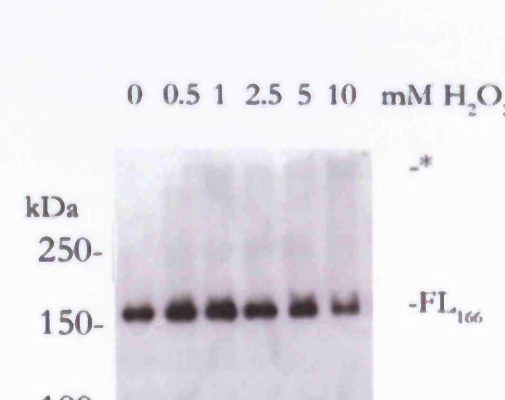
Wild type

B.



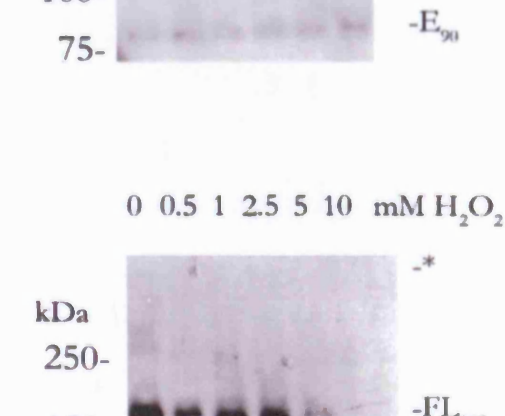
C215S Domain 1

C.



C215S Domain 2

D.



C866L

Figure 6.4: Effects of H_2O_2 on migration pattern of PTPσ peptides on non-reducing SDS-PAGE in the absence of iodoacetamide

HEK-293T cells cultured in F10 media with 10% foetal calf serum were transfected with wild type or mutant PTPσ (C215S domain 1, C215S domain 2, C866L). The cells were treated with hydrogen peroxide for 5 minutes at a range of concentrations. Before treatment, the serum-containing media was replaced with serum-free media. Cells were lysed in a buffer lacking iodoacetamide. The lysates were analysed using non-reducing SDS-PAGE. Western analysis used an antibody directed against the FLAG (amino-terminal) tag. PTPσ peptides are marked at the right hand edge of the figure. Equal total amounts (Bradford assay) of protein were loaded in each lane.

A. Wild type PTPσ
B. C215S domain 1 PTPσ
C. C215S domain 2 PTPσ
D. C866L PTPσ

* - High molecular weight species

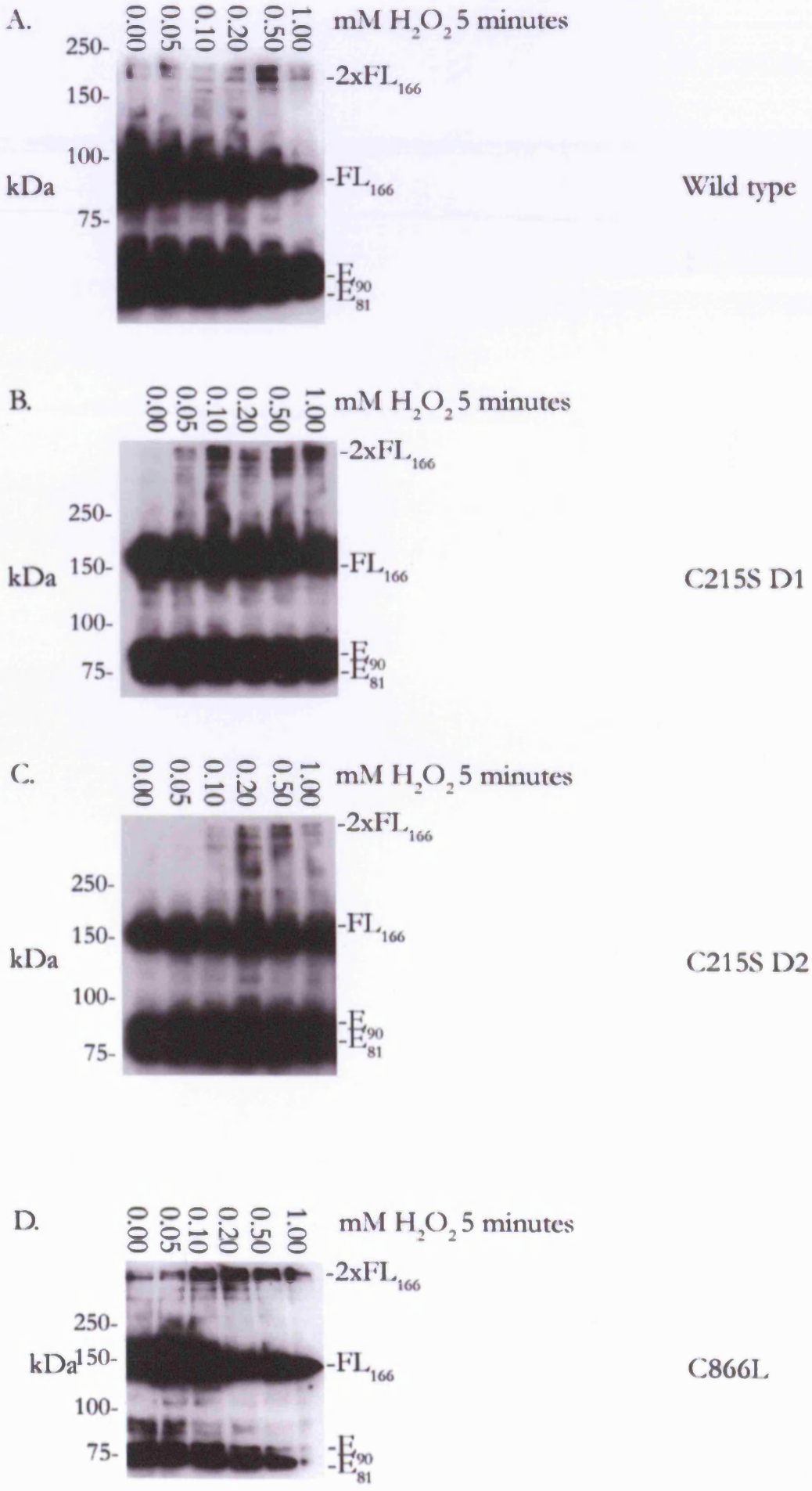

A.
0.00 0.05 0.10 0.20 0.50 1.00 mM H_2O_2 5 minutes
250-
150-
100-
kDa
75-
-2xFL166
-FL166
-E90
-E81
Wild type
B.
0.00 0.05 0.10 0.20 0.50 1.00 mM H_2O_2 5 minutes
-2xFL166
250-
kDa 150-
-FL166
100-
75-
-E90
-E81
C215S D1
C.
0.00 0.05 0.10 0.20 0.50 1.00 mM H_2O_2 5 minutes
-2xFL166
250-
150-
kDa
-FL166
100-
75-
-E90
-E81
C215S D2
D.
0.00 0.05 0.10 0.20 0.50 1.00 mM H_2O_2 5 minutes
-2xFL166
250-
kDa 150-
-FL166
100-
75-
-E90
-E81
C866L

Figure 6.5: H_2O_2 induces the formation of high molecular weight reduction sensitive aggregates from PTPσ expressing cells lysed in the presence of iodoacetamide.

HEK-293T cells cultured in F10 media with 10% foetal calf serum were transfected with wild type or mutant PTPσ (C215S domain 1, C215S domain 2, C866L). The cells were treated with hydrogen peroxide for 5 minutes at a range of concentrations. Before treatment, the serum-containing media was replaced with serum-free media. Cells were lysed in a buffer containing iodoacetamide (20mM). The lysates were analysed using non-reducing SDS-PAGE. Western analysis used an antibody directed against the FLAG (amino-terminal) tag. PTPσ peptides are marked at the right hand edge of the figure. Equal total amounts (Bradford assay) of protein were loaded in each lane.

A. Lysates from cells transfected with wild type PTPσ analysed by non-reducing SDS-PAGE and detected with an antibody directed against the FLAG (amino-terminal) tag.

B. Lysates from cells transfected with C215S Domain 1 PTPσ analysed by non-reducing SDS-PAGE and detected with an antibody directed against the FLAG (amino-terminal) tag.

C. Lysates from cells transfected with C215S Domain 2 PTPσ analysed by non-reducing SDS-PAGE and detected with an antibody directed against the FLAG (amino-terminal) tag.

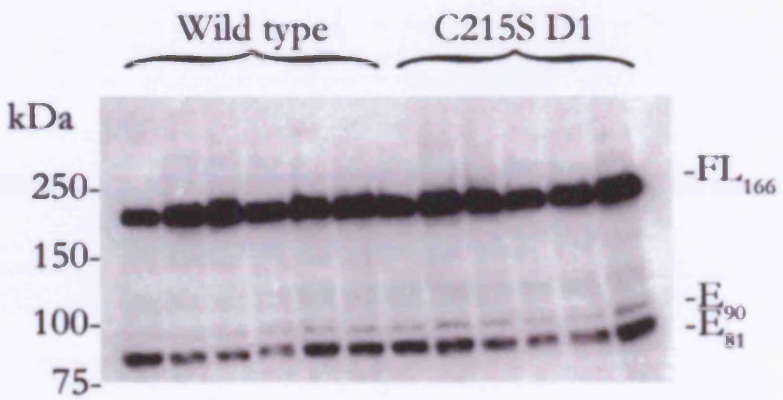
D. Lysates from cells transfected with C866L PTPσ analysed by non-reducing SDS-PAGE and detected with an antibody directed against the FLAG (amino-terminal) tag.

6.4: Treatment with foetal calf serum

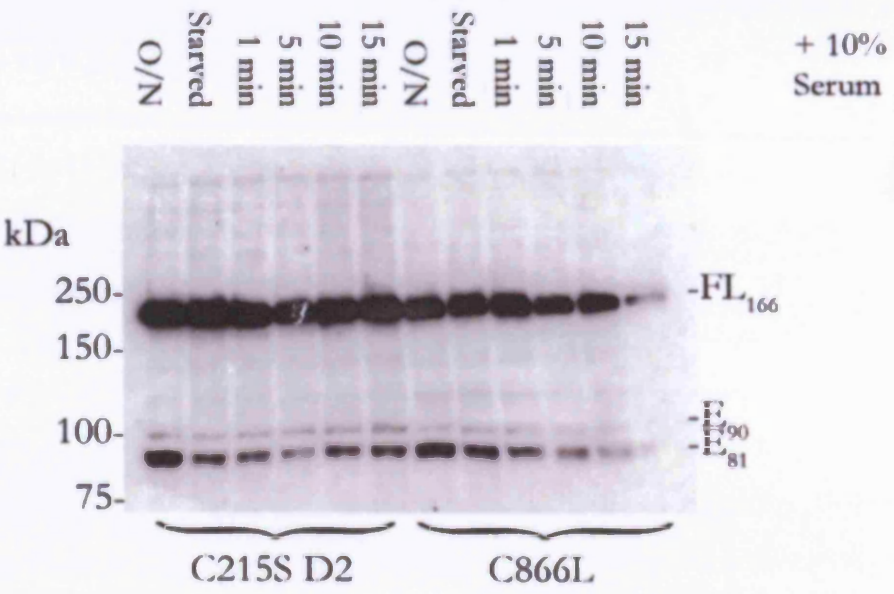
The generation of H_2O_2 in response to growth factor binding by receptor tyrosine kinases is one of the mechanisms by which RTKs activate their downstream pathways (Sundaresan et al., 1995; Bae et al., 1997; Meng et al., 2002). As H_2O_2 treatment causes PTPσ to form high molecular weight smears, it is of interest to determine whether a local, physiological source of H_2O_2, such as growth factor administration, has the same effect as the application of a much lower concentration over the whole cell. However, when wild type PTPσ-expressing cells were starved of serum overnight, they unexpectedly had a higher phosphotyrosine level than cells incubated overnight in serum-containing media (Figure 6.6C lanes 1, 2). Indeed, phosphotyrosine levels appeared to decrease in proportion to the duration of serum treatment (Figure 6.6C lanes 2-6). In contrast, cells that expressed catalytically inactive PTPσ (C215S D1) showed the expected picture; serum starved cells had lower phosphotyrosine levels than those incubated in serum overnight (Figure 6.6C, lanes 7-8). This effect was not seen with other cysteine mutations in PTPσ (C215SD2, C866L – Figure 6.6D), which implies that the catalytic activity of PTPσ is responsible for the decrease in cellular phosphotyrosine seen in serum treated cells. It may be noted that the cellular phosphotyrosine level decreased in proportion to the duration of serum treatment in all PTPσ-transfected cells. This may reflect a technical aspect of serum treatment that produces an apparent early decrease in cellular phosphotyrosine. One possible candidate is the contribution of cell associated serum proteins to the total protein content of the lysates. It was apparent from these results that although it had a paradoxical effect, cells expressing PTPσ responded to serum. Therefore, the effect of serum on high molecular weight formation was investigated.

Serum did not appear to affect high molecular species formation when PTPσ-expressing cells were lysed in an iodoacetamide-free buffer (Figure 6.6A-B). However, when cells were lysed in iodoacetamide-containing lysis buffer, serum treatment tended to increase high molecular weight species formation (Figure 6.7 A-D). Serum-induced high molecular weight species formation was not affected by mutations in the catalytic or transmembrane cysteines (C215S D1/2, C866L) and was thus qualitatively similar to the effect of H_2O_2. The effect of overnight growth in the presence of 10% serum was quantitatively similar to the effect of a 5 minute treatment in 1mM H_2O_2 (Figure 6.7).

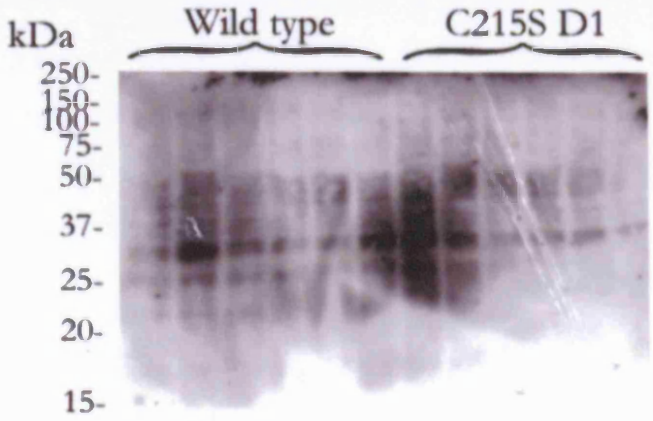
A.


Wild type
C215S D1
kDa
250-
150-
100-
75-
-FL166
-E90
-E81

B.


O/N
Starved
1 min
5 min
10 min
15 min
O/N
Starved
1 min
5 min
10 min
15 min
+ 10% Serum
kDa
250-
150-
100-
75-
-FL166
-E90
-E81
C215S D2
C866L

C.


kDa
Wild type
C215S D1
250-
150-
100-
75-
50-
37-
25-
20-
15-

D.

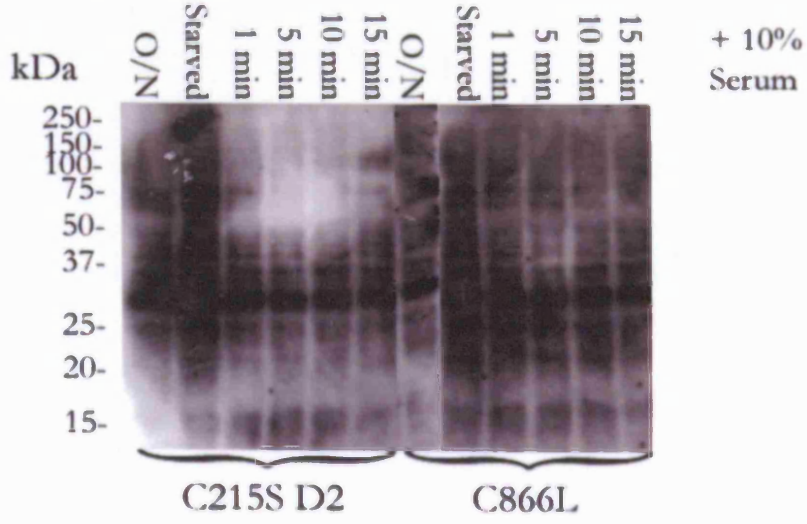

O/N
Starved
1 min
5 min
10 min
15 min
O/N
Starved
1 min
5 min
10 min
15 min
+ 10% Serum
kDa
250-
150-
100-
75-
50-
37-
25-
20-
15-
C215S D2
C866L

Figure 6.6: Effect of serum on PTPσ high molecular weight forms

HEK-293T cells cultured in F10 media with 10% foetal calf serum were transfected with wild type or mutant PTPσ (C215S domain 1, C215S domain 2, C866L). Overnight before the experiment the medium was replaced with serum- free media or had fresh serum containing media replaced. On the day of the experiment, cells were exposed to media containing 10% serum for the indicated length of time. Cells were lysed in a buffer lacking iodoacetamide. The lysates were analysed using non-reducing (A-B) or reducing SDS-PAGE (C-D). Equal total amounts (Bradford assay) of protein were loaded in each lane.

A. Non-reducing SDS-PAGE analysis of lysates from cells transfected with wild type (left) or C215S D1 (right) PTPσ detected with anti-FLAG antibody.
B. Non-reducing SDS-PAGE analysis of lysates from cells transfected with C215S D2 (left) or C866L (right) PTPσ detected with anti-FLAG antibody.
C. Reducing SDS-PAGE analysis of lysates from cells transfected with wild type (left) or C215S D1 (right) PTPσ detected with anti-phosphotyrosine antibody.
D. Reducing SDS-PAGE analysis of lysates from cells transfected with C215S domain 2 (left) or C866L (right) PTPσ detected with anti-phosphotyrosine.

6.5: Treatment with heparin

Serum contains an incompletely defined mixture of small molecules and proteinaceous growth factors. Thus, it may difficult to define which of these components are responsible for the results described above. There has been a great deal of interest in the role of heparan sulphate proteoglycans (HSPGs) as potential type IIa RPTP ligands and HSPG interactions have been identified for PTPσ and DLAR (Aricescu et al., 2002; Fox and Zinn, 2005). However, functional interactions have been difficult to prove.

Until this work, the effect of HSPG binding on PTPσ activity, cleavage, expression, localisation or oligomeric state remained undetermined. Treatment of PTPσ expressing HEK-293T cells with heparin did not affect PTPσ expression level, cleavage pattern or high molecular weight species formation (Figure 6.7). However, these experiments were prone to artefactual variations in the level of high molecular weight species seen. As noted in the figure legend, lane 1 on panel A and lane 1 on panel C (Figure 6.7) contain the same sample. However, on one transferred gel there is abundant high molecular weight signal, whereas on the other there is little high molecular weight signal. Similarly the apparent increase in high molecular weight signal seen between lanes 2 and lanes 6/7 on panel A (Figure 6.7) was not a reproducible finding.

There were several explanations for the lack of reproducibility of these experiments. Firstly, the total amount of high molecular weight species was always low and required prolonged exposure to detect it at all. Secondly, the location of the high molecular weight species at the edge of the gel made them susceptible to loss either if electrophoresis was not perfectly uniform between wells or if the stacking gel was not cleanly removed prior to transfer. In addition, there was some variability between each sample with regard to expression level or cell density. Finally, these large proteins transferred relatively poorly and as such were significantly affected by even small problems in the transfer process.

In summary, there was no consistent evidence that heparin affected the formation of high molecular weight species by PTPσ. However, given the importance of this question, it should certainly be reassessed using stably transformed cells and techniques including energy transfer and co-immunoprecipitation that are not as susceptible to the problems outlined above.

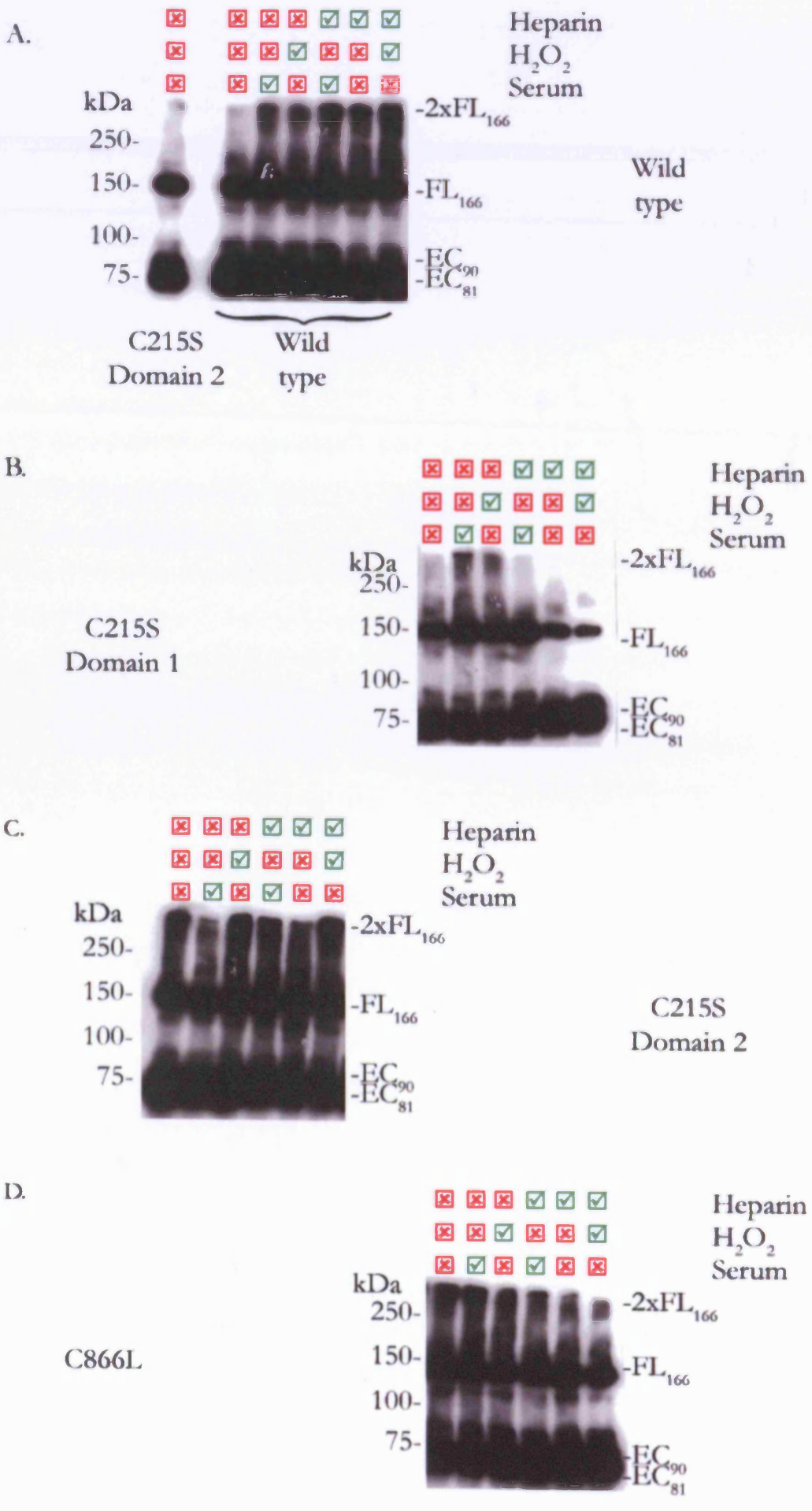

A.
Heparin
H_2O_2
Serum
kDa
250-
150-
100-
75-
-2xFL166
-FL166
-EC90
-EC81
Wild type
C215S Domain 2
Wild type
B.
Heparin
H_2O_2
Serum
C215S Domain 1
kDa
250-
150-
100-
75-
-2xFL166
-FL166
-EC90
-EC81
C.
Heparin
H_2O_2
Serum
kDa
250-
150-
100-
75-
-2xFL166
-FL166
-EC90
-EC81
C215S Domain 2
D.
Heparin
H_2O_2
Serum
C866L
kDa
250-
150-
100-
75-
-2xFL166
-FL166
-EC90
-EC81

Figure 6.7: Effect of heparin on high molecular weight species formation

HEK-293T cells cultured in F10 media with 10% foetal calf serum were transfected with wild type or mutant PTPσ (C215S domain 1, C215S domain 2, C866L) and lysed in the presence of iodoacetamide. Equal total amounts (Bradford assay) of protein were loaded in each lane. Treatments were performed as indicated above each lane, in the order listed below:

1. Manipulation of serum levels (serum-starved or F10+10% FBS overnight)
2. Heparin treatment (1μg/ml for one hour)
3. H_2O_2 treatment (1mM for 5 minutes)

Cells were lysed in a buffer containing iodoacetamide (20mM). The lysates were analysed using non-reducing SDS-PAGE. Western analysis used an antibody directed against the FLAG (amino-terminal) tag. PTPσ peptides are marked at the right hand edge of the figure.

A. Lysates from cells transfected with wild-type PTPσ
B. Lysates from cells transfected with C215S Domain 1 PTPσ
C. Lysates from cells transfected with C215S Domain 2 PTPσ
D. Lysates from cells transfected with C866L PTPσ

Lane 1 on panel A contains an equal amount of the same sample that was loaded into lane 1 on panel C. Panels A and B and panels C and D were run on the same gels and transferred and detected together.

6.6: Discussion

In the preceding chapters, this thesis has characterised several key features of PTPσ when it is overexpressed in human cells, namely the generation of cleavage products and oligomerisation. This chapter uses a number of biochemical reagents to determine what effect manipulation of specific pathways has on PTPσ.

Firstly, protein kinase C activation stimulates PTPσ cleavage and shedding as previously shown (Figure 6.1) (Aicher et al., 1997). Moreover, it is shown that PTPσ extracellular domain shedding can occur following carboxy-terminal cleavage alone, in the absence of amino-terminal cleavage. It may be suggested that shedding of the E_{90} fragment is a non-physiological response to the presence of large amounts of uncleaved protein. It is therefore notable that in some systems, extracellular domain shedding is seen not with wild type protein but only with mutations that reduce the level of amino-terminal cleavage (Serra-Pages et al., 1994). However, shedding of both extracellular fragments shows a dose responsive increase to PKC activation, which might not be expected if the non PKC stimulated amino-terminal cleavage event were acting as a limiting factor.

In previous reports, only one PTPσ band was shed in response to TPA. However, studies on LAR have shown the shedding of two species in response to TPA (Serra-Pages et al., 1994; Pulido et al., 1995a). The differences between this and previous studies on PTPσ may reflect the use of different cell lines or the choice of an alternative time-point (40 hours post-transfection) at which to carry out the treatment. It is also possible that transfection methods using calcium interfere with ectodomain shedding or the distribution of probe between media and cell-bound populations (Aicher et al., 1997). However, in my hands, untreated cells analysed at 40 hours shed only the E_{81} form and do not express the E_{90} species (Chapter 3). In previous studies, which only reported the shedding of a single PTPσ band, it is difficult to be certain whether the shed species represents E_{90} or E_{81}. Few studies have directly compared the size of shed protein with that of cell-associated protein. There is indirect evidence that this solitary protein species is the product of carboxy-terminal cleavage and not amino-terminal cleavage. Firstly, the resulting intracellular domain fragment sequence starts at the carboxy-terminal not the amino-terminal cleavage site. Although this would also be the case if the protein had undergone cleavage at both the amino- and carboxyl-terminal sites, the molecular weight of the cell-associated extracellular domain fragment either decreases in size or stays the same following TPA or calcium ionophore treatment. Both of these treatments are associated with ectodomain shedding.

As only the extracellular domain fragment resulting from carboxy-terminal cleavage can become smaller by subsequent cleavage at the amino-terminal site, this might suggest that where only one band is seen, it might represent E_{90} and not E_{81}. Therefore, this is significant reason to believe that the extracellular domain of type II RPTPs can be shed following cleavage at only the carboxy-terminal site. It is however unclear whether amino-terminal cleavage is also required for extracellular domain shedding. It may in the future be informative to subject cells expressing the disulphide crosslinking mutants to TPA treatment. This may reveal whether the shed ectodomains are dimeric as the larger shed fragment should contain the ectopic cysteine residue and hence retain the ability to form disulphide crosslinked dimers (4.2.2).

Secondly, the inhibition of cysteine-based tyrosine phosphatases with pervanadate has no effect on PTPσ cleavage or expression level (Figure 6.2). Pervanadate treatment irreversibly inactivates all cysteine-based tyrosine phosphatases, preventing tyrosine dephosphorylation. In addition, because many tyrosine kinases are activated by active site loop tyrosine phosphorylation, pervanadate significantly increases cellular tyrosine kinase activity. PTPσ becomes phosphorylated on tyrosine residues in response to pervanadate. However, the cleaved PTPσ intracellular domain becomes significantly more highly phosphorylated than full-length protein. It has not yet been possible to determine whether it is only one or both of the intracellular domain fragments that become phosphorylated in this way. This suggests a link between phosphorylation state and proteolytic cleavage. As an increase in tyrosine phosphorylation does not induce post-translational cleavage, it is likely that cleavage makes the intracellular domain more available to protein kinases than the full-length protein. The most likely mechanism for this observation is that cleaved protein occupies a different cellular compartment than full-length protein. Such a phenomenon has previously been documented following PKC activation, however it remains technically difficult to distinguish cleavage products from full-length protein within intact cells. Alternatively, either cleavage or phosphorylation state may have a conformational effect on PTPσ affecting the recognition of the various species by the immunoprecipitating or detection antibody. Indeed, cleavage-associated conformational changes might in turn affect the accessibility of tyrosine residues to kinase-mediated phosphorylation. However, in this case the marked difference in phosphorylation state of cleaved and full-length protein does not seem to be matched by a similar difference in the amount of each protein pulled down by the immunoprecipitating antibody. Therefore, the basis for this observation remains undetermined. Nevertheless, it represents a possible method for

tracking changes in PTPσ location and possibly functional state. Finally, pervanadate did not have any effect on the formation of high molecular weight species formation by PTPσ.

In contrast, treatment with a less potent tyrosine phosphatase inhibitor, H_2O_2, initially failed to have any effect on cellular phosphotyrosine levels (Figure 6.3E). It was shown that the experimental conditions for H_2O_2 treatment were more fastidious than those for pervanadate treatment. Under optimised conditions, H_2O_2 treatment also increased cellular phosphotyrosine levels, although as expected this effect was more modest than that seen with pervanadate (Figure 6.3F). Although H_2O_2 did not appear to affect PTPσ cleavage, H_2O_2 treatment seemed to be associated with a reduction in overall PTPσ level that was proportionate to H_2O_2 concentration. Moreover, H_2O_2 treatment resulted in the appearance of high molecular weight species similar to those reported in Chapter 4 (Figure 6.3-5). In that chapter, it was concluded that as these species were seen only on non-reducing analysis and were affected by cysteinyl modifying reagents they were likely to represent disulphide-bonded complexes of PTPσ. Formally, such complexes may form between PTPσ molecules or between individual PTPσ molecules and other cysteine-containing proteins. The high molecular weight species seen following H_2O_2 treatment are similar in size to the disulphide bonded dimers described in chapter 4, and the formation of disulphide-bonded dimers in response to H_2O_2 has been described for the related PTPα (van der Wijk et al., 2004). Therefore, it is likely that the reduction-sensitive high molecular weight species represent disulphide bonded PTPσ dimers. However, there are distinct features of high molecular weight species formation by PTPσ depending on the presence or absence of iodoacetamide in the lysis buffer.

In the absence of cysteinyl-modifying reagents, H_2O_2 increased disulphide bond formation in wild-type PTPσ-expressing cells (Figure 6.4). However, this effect was less marked in cells expressing PTPσ with any cysteine mutation. In the presence of cysteinyl modifying reagents, although H_2O_2 also increased disulphide bond formation, the high molecular weight species had distinct characteristics from those seen in the absence of cysteinyl modifying reagents (Figure 6.5). In contrast to the diffusely smeared high molecular weight species seen in the absence of cysteinyl-modifying agents, at least two clearly distinct bands could be seen in lysates containing cysteinyl-modifying agents. These high molecular weight species were seen in lysates from cells expressing wild type PTPσ and in lysates from cells expressing mutant PTPσ (C215S D1/2, C866L). In addition, in lysates from cells expressing wild type, C215S D1 or C866L PTPσ the total amount of detectable PTPσ decreases with increasing H_2O_2 concentration. This effect was less

apparent in lysates expressing PTPσ with the C215S D2 mutation. Finally, H_2O_2 treatment appeared to be associated with a reduction in overall PTPσ level both in the presence and absence of iodoacetamide. It is necessary to consider in turn the formation of disulphide bonded high molecular weight species in the presence and absence of cysteinyl modifying reagents. In addition, the significance of the reduction in overall PTPσ level seen on H_2O_2 treatment will be discussed.

The formation of high molecular weight species in the absence of cysteinyl modifying reagents is likely to reflect the formation of non-physiological disulphide bonds after cell lysis and during denaturation under non-reducing conditions. In untreated cells expressing wild type PTPσ this effect is blocked by the addition of an excess of a cysteinyl-modifying reagent. Use of a chemical such as iodoacetamide in the lysis buffer would have two effects. It would not affect any disulphide bonds formed before cell lysis but it would significantly reduce post-lysis disulphide bond formation. This would include the interaction of previously buried, unpaired cysteine residues that are exposed by protein denaturation under non-reducing conditions. In addition, such reagents would inactivate all enzymes that rely upon cysteine residues for their catalytic mechanism; this would include classical tyrosine phosphatases and cellular cysteine reducing systems. The formation of disulphide bonds under these conditions may be considered in two categories. Firstly, disulphide bonds may form from the random collision of proteins containing free cysteines. Such interactions would produce a smear of disulphide-bonded species. Although a smear was seen under these conditions, such an effect would also be seen with a variety of species that differ in the location of their disulphide bond. Such a situation would also result if the disulphide bonds formed were not stable, being reduced either by their local environment or by the action of solubilised cysteine-reducing enzymes. However, disulphide bonds produced by the random collision of molecules in solution would not be significantly affected by point mutations in specific cysteine residues unless that cysteine residue was responsible for a significant proportion of a protein's free cysteine content. The formation of high molecular weight PTPσ-containing species in the absence of iodoacetamide was susceptible to point mutations in any of three cysteine residues. Given that each of the mutated cysteines must be close enough to interact, mutations at these sites might perturb the formation of the dimer. Moreover, all three residues are likely to be surface-exposed rather than buried at the centre of a hydrophobic domain and only becoming accessible after denaturation. Therefore, it is unlikely that these disulphide-bonded interactions reflect the random interactions of denatured proteins in solution.

Moreover, other researchers have been unable to identify any high molecular weight smears in the absence of cysteinyl modifying reagents even using PTPα. PTPα contains a cysteine-rich domain that might be expected to contribute to the formation of high molecular weight aggregates if this effect really occurs. However, PTPα does not form reduction sensitive high molecular weight smears in the absence of cysteinyl modifying reagents (van der Wijk et al., 2004). Indeed it is also impossible to produce high molecular weight forms with disulphide cross-linking PTPα mutants in the absence of cysteinyl modifying reagents. This is attributed to the extreme sensitivity of such bonds to cellular disulphide reductases (Jiang et al., 1999). Finally, with regard to the formation of high molecular weight species by PTPσ, significantly greater amounts of high molecular weight species were seen in lysates containing Triton X-100 than in those containing Nonidet P-40. Similarly, pre-lysis treatment of cells with H_2O_2 increased high molecular weight species formation. It therefore seems that the underlying mechanism for high molecular weight species formation occurs pre-lysis. Therefore, there may be some degree of specificity to the aggregation seen in the absence of cysteinyl reducing agents and a more likely hypothesis is that disulphide bond formation by PTPσ, in the absence of cysteinyl modifying reagents, reflects the oxidation of closely approximated cysteines in a preformed dimer.

The formation of disulphide bonds in the presence of cysteinyl modifying reagents is likely to represent pre-lysis disulphide bond formation. The increased abundance of disulphide bonded forms of PTPσ and their increased resolution on non-reducing analysis is likely to be due to the inactivation of cellular reducing enzymes. Such enzymes might otherwise lead to fluctuations in the total level of disulphide-bonded species and in the oligomeric state of individual molecules leading to the diffuse low-level signal seen in the absence of iodoacetamide. The use of a high concentration of iodoacetamide in the lysis buffer and its continuing presence in the sample buffer used for protein denaturation should mask all free cysteines in the sample. Therefore, the disulphide-bonded species seen in these experiments must reflect either disulphide bonds that form within the cell or very rapidly after lysis. As such, they are likely to have formed between closely apposed proteins. Thus, their formation may be an aspect of the physiological regulation of RPTPs. Alternatively, their formation may be artefactual reflecting an interaction between extremely closely apposed cysteine residues in a favorable local environment. However, even if this latter hypothesis were true, it is unlikely that such disulphide bonds would form *in vivo,* where cellular reducing enzymes would continually reduce them. In either case, it can be argued that changes in the level of disulphide bonded species formation might

reflect changes in the proportion of dimeric PTPσ or in the conformation of PTPσ monomers in preformed dimers. If high molecular weight smear formation is a measure of dimer formation, it may be a more accurate measure of PTPσ dimerisation at the moment of cell lysis than co-immunoprecipitation. Moreover, it would not be susceptible to any effect that dimerisation or oxidative stress might have on epitope accessibility (van der Wijk et al., 2003).

It is notable that disulphide bond formation in the presence and absence of cysteinyl modifying reagents shows different characteristics than that seen with the related PTPα (van der Wijk et al., 2004). Firstly, formation of disulphide-bonded dimers by PTPα requires the catalytic cysteine in both catalytic domains. The formation of PTPσ disulphide bonded dimers does not require the first catalytic site cysteine and some disulphide bond formation is seen in mutants lacking the second catalytic domain cysteine. Unfortunately, time did not permit the assessment of disulphide bond formation in mutants lacking both PTPσ active site cysteines. Secondly, only a small proportion of PTPσ is found in the disulphide bonded dimeric form following H_2O_2 treatment and long exposures are required to detect it. In contrast, PTPα disulphide bonded dimers represent a significant proportion of total cell PTPα following H_2O_2 treatment. These may reflect genuine differences in the mechanisms of PTPα and PTPσ disulphide bond-mediated dimerisation. Alternatively, there may be differences in expression level or in the oligomeric state of the two RPTP molecules at rest. These may result in different factors limiting disulphide bond formation for each protein.

In addition to inducing high molecular weight species formation, it appeared that H_2O_2 treatment led to a decrease in the total PTPσ protein level. It must be noted that accurate quantification of the magnitude of any effect of H_2O_2 was complicated by several factors. It was not possible to perform these experiments using cell lines stably transfected with wild type or mutant PTPσ. Therefore, the experiments were performed by splitting large plates of PTPσ-transfected cells into multi-well plates for H_2O_2 treatment. However, even using this method it was still impossible to obtain completely even expression levels and patterns of cleavage across all the wells. Therefore, it will be interesting to repeat these experiments using either a cell line that expresses endogenous levels of PTPσ or that has been stably transfected with PTPσ. In addition, there may be localised fluctuations in transfer efficiency across a large gel, which may be especially significant when comparing relatively small changes in the level of high molecular weight species. However, the decrease in total PTPσ level seen in response to H_2O_2, was consistent across multiple

independent experiments and so is likely to represent a real effect. There are several possible explanations for this effect. Most importantly, if H_2O_2 increases high molecular weight species formation, then a proportion of the FL_{166} species will be converted into the larger $2xFL_{166}$ dimers, which transfer less efficiently. However although the decrease in total protein level is seen with wild type PTPσ and in C215S D1 and C866L mutant PTPσ, lysates from cells expressing the C215S D2 mutant protein do not show an equivalent decrease in total PTPσ protein. This mutant also shows reduced levels of high molecular weight species formation in response to H_2O_2 (in the presence of iodoacetamide). This residue has previously been shown to be a key mediator of the response of PTPα to H_2O_2 (Blanchetot et al., 2002a; van der Wijk et al., 2003; van der Wijk et al., 2004; Persson et al., 2004; Groen et al., 2005). Therefore, although interpretation of the effect of H_2O_2 under these conditions is complicated, there are suggestions that PTPσ behaves in a similar way to PTPα.

Treatment of serum starved PTPσ-transfected cells with foetal calf serum had no effect on PTPσ cleavage or expression level. However, when serum-treated cells expressing PTPσ were lysed in the presence of iodoacetamide, serum was seen to increase high molecular weight species formation (Figure 6.6A). Foetal calf serum is a rich source of growth factors. Stimulation of growth factor tyrosine kinase receptors is associated with local hydrogen peroxide generation, which is believed to potentiate tyrosine kinase signaling by inactivating nearby tyrosine phosphatases. Thus the application of H_2O_2 to cells may mimic the effect of H_2O_2 generation in response to growth factor signaling. However, the degree of variability seen in these experiments means that further work is necessary before any conclusions are drawn. This may include using defined growth factors whose receptors have been shown to interact genetically or biochemically with PTPσ.

In addition, treatment of PTPσ-transfected cells with foetal calf serum produced a unexpected decrease in cellular phosphotyrosine (Figure 6.6B). It is likely that serum treatment of untransfected HEK-293T cells will produce an increase in cellular phosphotyrosine; this has previously been documented in other cell lines. However, the effect of serum on untransfected HEK-293T cells was not determined during these studies. The assumption that serum treatment will increase cellular phosphotyrosine in untransfected cells is further supported by the response of cells expressing catalytically inactive PTPσ (C215S D1) to serum treatment. Cells transfected with C215S D1 PTPσ show an increase in cellular phosphotyrosine in response to serum treatment. On the other

hand, cells transfected with the C215S D2 or C866L mutant PTPσ show a decrease in cellular phosphotyrosine in response to serum treatment.

It is not clear why PTPσ expression should reverse the normal effect of serum on tyrosine phosphorylation. The requirement for PTPσ catalytic activity suggests that serum treatment has a direct stimulatory effect on PTPσ activity. Furthermore, it is apparent that the effect of serum on PTPσ phosphatase activity does not involve significant changes in PTPσ cleavage or expression level. Changes in PTPσ sub-cellular localisation were not assessed and might be responsible for the affect on PTPσ phosphatase activity by altering the access of the enzyme to a range of substrates, phosphorylated in response to serum activation. Alternatively, serum treatment might increase PTPσ tyrosine phosphatase activity directly either via the induction of PTPσ tyrosine phosphorylation on a positive regulatory site, or by the recognition of a specific PTPσ ligand in serum or by the presence in serum of a factor that reduces the binding of a culture-derived inhibitory PTPσ ligand. It is indeed unclear whether the activity of RPTPs is regulated by ligand binding in a manner analogous to RTK activity and the available evidence implies that there may be both stimulatory and inhibitory ligands. It is moreover unclear whether the effect of serum on RPTP-transfected cells is specific to PTPσ, suggesting a PTPσ-specific factor such as a ligand. Alternatively, the effect seen might reflect a general effect of serum on the activity of all RPTPs. This might reflect a generalised activation of available PTPs following the widespread RTK activation induced by serum. A picture of this kind might suggest that PTPs are positively regulated by their own tyrosine phosphorylation state. Therefore, it will be extremely interesting to identify the mechanism responsible for the effect of catalytically active PTPσ expression on the cellular response to foetal calf serum.

The only known PTPσ ligand, heparin, has no effect on PTPσ expression level, cleavage or high molecular weight species formation (Aricescu et al., 2002) (Figure 6.7). Unfortunately, the effect of heparin on cellular phosphotyrosine was not determined. Other type II RPTPs also interact with HSPGs with a varying effect on tyrosine phosphatase activity and it is becoming apparent that regulation with HSPGs might be a common feature of type II RPTPs (Fox and Zinn, 2005). The absence of a clear result is difficult to interpret. It is possible that treatment of PTPσ-expressing cells with heparin has no effect. However, it is also possible that an inappropriate concentration of Heparin has been used. The dose and incubation time selected does result in changes in retinal ganglion cell neurite outgrowth (G.Sajnani personal communication). However, overexpression often significantly perturbs cellular signaling pathways and may alter the reaction kinetics.

Specifically, the higher level of receptor expression in an overexpression system when compared to an endogenously expressing cell model may require the use of much higher concentrations of ligand in the former before effects are seen. Lastly, it may be possible that heparin is having an affect on PTPσ protein in these cells, but that this does not result in a phenotypic change that we are able to detect. Alternatively, the media used in these experiments may contain sufficient heparin to exert a maximal effect on PTPσ without addition of exogenous heparin (Wang et al., 2000). Therefore, it will be necessary in future to dissect the relationship between heparin and PTPσ by using different cell lines, assays and treatment parameters. In particular, it will be necessary to examine the effect of heparin on the expression level, cleavage pattern and high molecular weight species formation in a cell model in which heparin treatment has an effect (Sajnani et al., 2005).

In this chapter, several key characteristics of PTPσ were examined using biochemical treatments and mutants. The effects of posttranslational cleavage of PTPσ at different sites were distinguished using techniques that suggest that the canonical model for PTPσ processing does not completely explain the available data. Moreover, it was shown that tyrosine phosphorylation is unlikely to be a factor in PTPσ cleavage. However, cleavage has significant effects on PTPσ tyrosine phosphorylation probably via the previously reported changes in PTPσ sub-cellular localisation. Such data also suggest that the different processed PTPσ species may have significantly different functions. Furthermore, an effort was made to identify the factors that regulate PTPσ dimerisation in vivo. Although H_2O_2 treatment increases PTPσ dimerisation, *in vivo* application of stimuli that produce physiological levels of intracellular H_2O_2 failed to have any affect on PTPσ dimerisation. However, it does not appear that the only identified PTPσ ligands, HSPGs, have their as yet undetermined effect by altering the pattern of PTPσ dimerisation, expression level or cleavage pattern. It is likely that further experiments that more precisely determine the molecular function of PTPσ will be important in discovering the function and regulatory mechanisms of PTPσ. In addition, various strategies will allow the identification of further PTPσ interacting proteins.

Chapter 7: Analysis of PTPσ-Interacting proteins

The work described above focused on the interactions of PTPσ with itself. Whilst the formation of oligomeric PTPσ complexes is likely to be very important for its mechanism of action, PTPσ function may be better described by identifying the other proteins, including substrates and ligands that interact with PTPσ. This thesis will separate the proteins that interact with PTPσ into three categories: ligands, interacting partners and substrates. For the sake of clarity, only those proteins that interact with the extracellular domain of PTPσ will be considered PTPσ ligands. In this sense, ligands need not cause a detectable change in PTPσ function. Similarly, only tyrosine-phosphorylated proteins that can be de-phosphorylated by the PTPσ phosphatase domain will be considered PTPσ substrates. Lastly, interacting partners are those proteins that interact with regions other than the extracellular domain and that are not subject to PTPσ-mediated dephosphorylation.

In this chapter, several pieces of work are described that attempt to identify PTPσ interacting proteins. Firstly, the 3xFLAG epitope used throughout this thesis was shown to have a negligible effect on the binding of a PTPσ-alkaline phosphatase fusion protein to known ligand-bearing tissues. Given the work described in previous chapters, which showed that PTPσ molecules can form *cis*-dimers when expressed in the same cell, it was important to assess whether PTPσ can also form dimers when presented *in trans*. The ability of the PTPσ ectodomain to bind homophilically was reassessed using an alternative technique (Haj et al., 1999). However, only very small amounts of the PTPσ extracellular domain bound to PTPσ-expressing cells. Thus, in contrast to the type IIb RPTPs, PTPσ may not act as a homophilic adhesion molecule (Brady-Kalnay et al., 1993; Gebbink et al., 1993b; Brady-Kalnay and Tonks, 1994; Sap et al., 1994; Zondag et al., 1995; Wang and Bixby, 1999; Baker et al., 2000b; Yang et al., 2003; Cismasiu et al., 2004; Yang et al., 2005; Aricescu et al., 2006). A series of His-tagged PTPσ extracellular domain deletion proteins was designed to investigate PTPσ extracellular domain structure and to identify PTPσ ligands. Some of these deletions showed detergent-resistant dimerisation in SDS-PAGE, suggesting that the PTPσ extracellular domain alone retains some ability to dimerise. The structure and ligand-binding properties of these proteins remain to be characterised.

Secondly, co-immunoprecipitation techniques using wild type PTPσ failed to detect any interacting proteins above background. Using PTPσ trapping mutations that enrich immunoprecipitates for PTPσ substrates, low levels of possible PTPσ substrates were

detected as previously reported (Aricescu, 2002). Unfortunately, it was not possible to obtain sufficient amounts of these putative substrates to allow their biochemical identification. In order to scale up this purification, a number of PTPσ intracellular domain Glutathione-S-Transferase (GST) fusions were created, both of wild-type PTPσ and of substrate-trapping PTPσ. The expression of these constructs was demonstrated; however, time did not permit the isolation of putative PTPσ substrates using these proteins.

7.1: Extracellular domain interactions

It has proved difficult to identify functional ligands for any RPTP, including PTPσ. Although a number of PTPσ ligands have been identified, the identification of any effect on PTP function has proven elusive. PTPσ binds at least two ligands in the developing chick and also acts as one of several receptors for the Black widow venom toxin α-latrotoxin (Aricescu et al., 2002; Krasnoperov et al., 2002; Sajnani-Perez et al., 2003). One of the ligands in developing chick embryos, which is located in basement membranes, is a heparan-sulphate proteoglycan (HSPG). In vitro, PTPσ binds a number of HSPGs including collagen 18 and agrin (Aricescu et al., 2002). Whether PTPσ binds a single or a range of HSPGs in these tissues is not yet clear. A second PTPσ ligand is located in muscle and does not involve heparan sulphate chains (Sajnani-Perez et al., 2003). This ligand is likely to be a cell-surface form of the nucleolar protein, nucleolin, and the significance of this ligand-receptor interaction is currently being investigated (Alete et al., 2006). It has not yet been possible to demonstrate a functional effect of binding of either ligand. In addition, PTPσ has recently been shown to act as a substrate for neurite outgrowth of retinal ganglion cell axons (Sajnani et al., 2005). This implies that PTPσ can act as a ligand as well as a receptor. The interaction of PTPσ with the neurite expressed protein has similarities to the interaction of PTPσ with the muscle ligand, being affected by the same amino acid mutations and being unaffected by heparinase treatment. It is therefore unclear whether this interaction reveals a third class of PTPσ ligand or utilises one of the previously identified ligands.

Both the type IIa RPTPs, PTPδ and LAR, and the related type IIb RPTPs have isoforms that exhibit homophilic binding both in *cis* and in *trans* (Brady-Kalnay et al., 1993; Gebbink et al., 1993b; Brady-Kalnay and Tonks, 1994; Sap et al., 1994; Zondag et al., 1995; Wang and Bixby, 1999; Baker et al., 2000b; Yang et al., 2003; Cismasiu et al., 2004; Yang et al., 2005; Aricescu et al., 2006). The data shown in previous chapters demonstrated that PTPσ can interact in *cis* to form oligomeric complexes. However, it was not possible to

determine whether PTPσ molecules can also interact in *trans* in mammalian cells. However, in co-immunoprecipitation experiments data was obtained that suggested that solubilised PTPσ also interact weakly in solution after cell lysis, suggesting the possibility of a *trans* binding mechanism. Therefore, PTPσ must still be considered a potential ligand for itself. Experiments were performed to determine whether PTPσ is in fact capable of interacting in this way.

7.1.1: Effects of FLAG epitope on PTPσ ligand binding

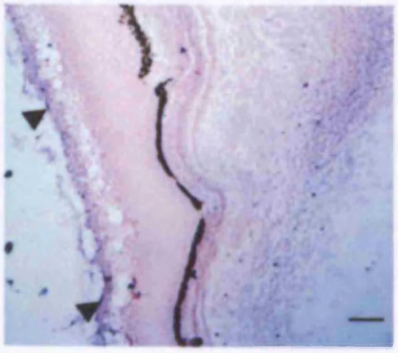
Much of the work described in this thesis uses 3xFLAG-tagged PTPσ proteins. This epitope tag is located at the extreme amino-terminal end of the protein. The PTPσ proteins used do not lack any endogenous sequence other than the signal peptide. However, due to the location of epitope tag, it is possible that it may interfere with ligand binding. Indeed a major determinant of the heparan-sulphate binding site is located close to the amino-terminus, in the first Ig domain (Aricescu et al., 2002). Therefore, experiments were performed to determine whether the addition of the 3xFLAG tag interfered with PTPσ binding to the known ligands in basement membrane and muscle.

The 3xFLAG tag does not interfere with PTPσ ligand binding. Previous work has characterised PTPσ ligand binding using receptor-alkaline-phosphatase (RAP) fusions (Haj et al., 1999; Flanagan and Cheng, 2000; Flanagan et al., 2000; Sajnani-Perez et al., 2003). A PTPσ-AP fusion protein was created that included the 3xFLAG epitope tag located amino-terminal to the PTPσ1 extracellular domain. The binding pattern of this probe (3xFLAG-PTPσ1-EC-AP, 3xFLAG probe; Figure 7.1 E-H) was compared to that of a similar probe lacking only the 3xFLAG tag (PTPσ1-EC-AP, wild type probe; Figure 7.1 A-D).

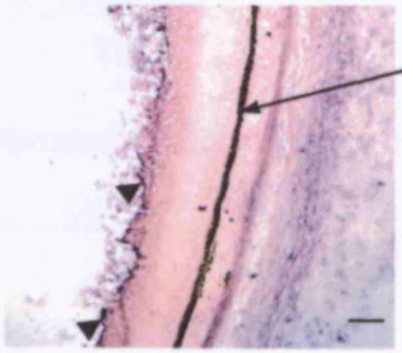
The 3xFLAG probe exhibited similar binding to that previously described for the wild type probe (Haj et al., 1999; Sajnani-Perez et al., 2003); Unpublished data, A. Stoker). It should be noted that the binding pattern is weaker than that previously published as previous work has predominantly utilised the FNΔ3 deletion which has the strongest binding of all PTPσ deletion mutants (Chilton, 2000). In the eye at embryonic day 8 (E8), the 3xFLAG probe binds to several outer retinal cell layers with particularly strong binding in regions where the retinal inner limiting basement membrane is intact (Arrowheads, Figure 7.1 A, E). Within the head, binding is also seen to skeletal muscle fibres (Figure 7.1 B,F) and to the epithelia of the developing oral and nasal cavity, most particularly to the epithelial basement membrane (Figure 7.1 D,H). The pattern of muscle staining has

previously been described and there is no detectable difference between the binding of the 3xFLAG probe and the wild type probe (Haj et al., 1999; Sajnani-Perez et al., 2003). In addition, PTPσ RAP probes show strong binding to the basement membrane in developing feather buds and the epidermis of the skin (Figure 7.1 C, G). The pattern of PTPσ RAP probe binding to feather buds is distinctive both in longitudinal and tranverse section. In longitudinal section, staining increases towards the tip of the feather (Figure 7.1 C, G). In transverse section, a striking star-shaped pattern of staining is seen (Figure 7.1 C inset, G inset). This pattern suggests that a ligand is present between the basilar epidermis and pulp epithelium of the developing feather (Yu et al., 2004). The binding of PTPσ probes to the basement membrane of skin and its derivatives (feathers) has not previously been published but is well known within the field (Unpublished data, A. Stoker).

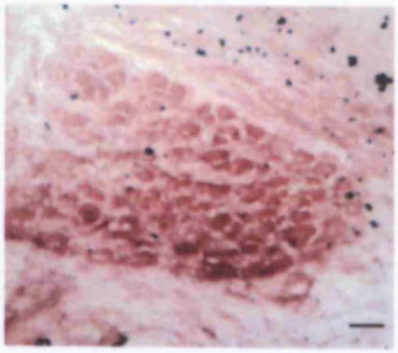
A.



E.



B.



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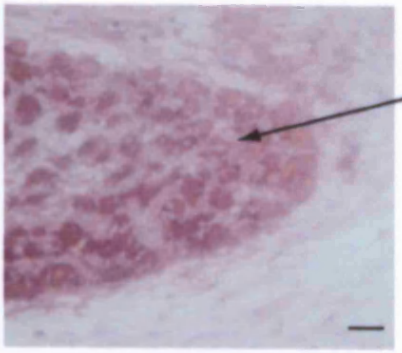
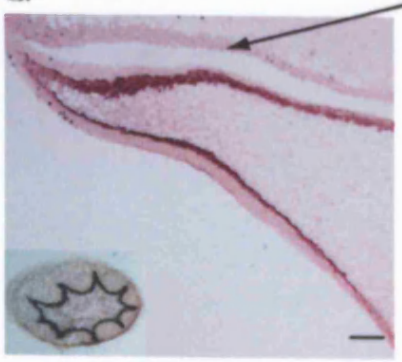
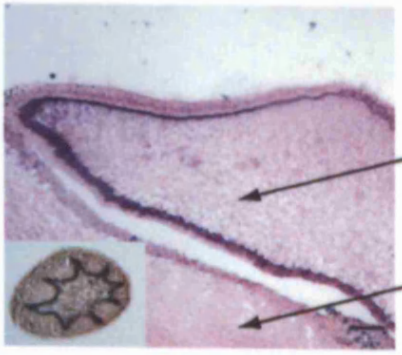


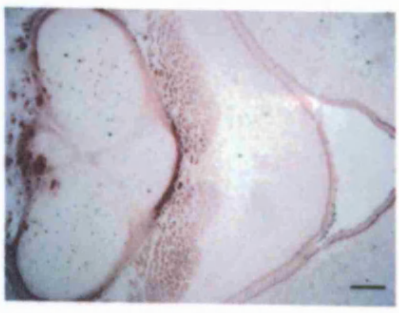
C.



G.



D.



H.

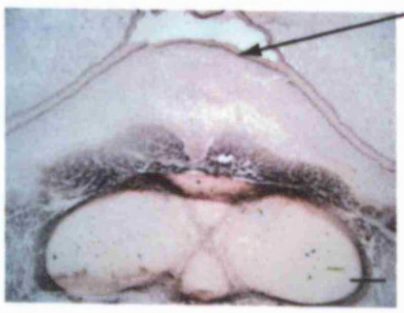


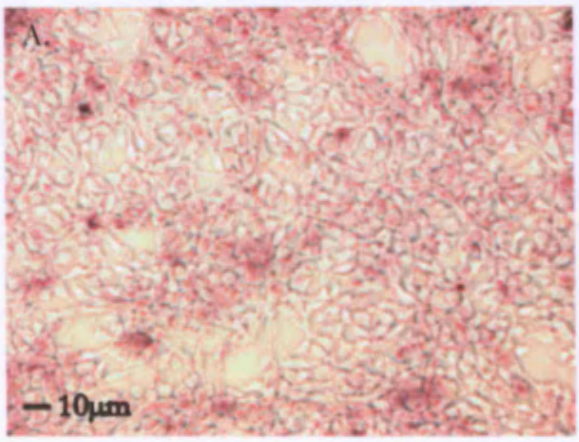
Figure 7.1: Effect of 3xFLAG epitope tag on PTPσ1 ligand binding in the RAP assay

Localisation of PTPσ1 ligands at embryonic day 8. RAP assays were carried out using PTPσ-EC-AP (A-D) or 3xFLAG-PTPσ1-EC-AP (E-H) on sections of E8 retina (A, E), extraocular muscle (B, F), developing feather buds (C, G) and beak (D, H). Insets in C and G show cross-sectional profile of RAP binding in feather buds. Sites of ligand interaction stain purple/blue. RPE, retinal pigmented epithelium; M, muscle; FB, feather bud; BW, body wall; ED, epidermis; Ep, Oropharyngeal epithelial basement membrane. Arrowhead indicates regions of basement membrane in the retina (A, E). Magnification was 50x (D, H), 100x (A, E, C, G) and 400x (B, F). Scale bars are included in each figure and represent 40μm (Panels A, E, C, G), 10μm (Panels B, F) and 80μm (Panels D, H).

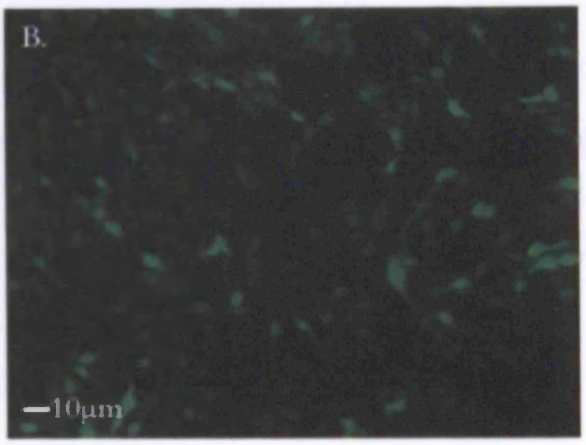
7.1.2: PTPσ Homophilic binding assays

Although PTPσ is believed to bind HSPGs and nucleolin, other ligands have not been identified (Aricescu et al., 2002; Alete et al., 2006). Many adhesion factors and several RPTPs exhibit homophilic binding, which can occur either when the protein of interest is expressed on different cells (in *trans*) or when both interacting proteins are present in the same membrane (in *cis*) (Brady-Kalnay et al., 1993; Gebbink et al., 1993b; Brady-Kalnay and Tonks, 1994; Sap et al., 1994; Zondag et al., 1995; Wang and Bixby, 1999; Baker et al., 2000b; Yang et al., 2003; Cismasiu et al., 2004; Yang et al., 2005; Aricescu et al., 2006). Overexpression of a soluble vesicular stomatitis virus (VSV)-tagged PTPσ extracellular domain throughout the avian retina results in an accumulation of protein only in the region of the PTPσ retinal basement membrane ligand (Rashid-Doubell et al., 2002). This suggests that PTPσ does not form trans-dimers or function as a homophilic adhesion molecule. However, lysis of samples of embryonic chick tissues yields only cleaved PTPσ suggesting that in these tissues PTPσ is rapidly cleaved and shed from the cell surface. Therefore, these assays do not reveal the existence or nature of any interaction between individual cleaved PTPσ extracellular domains or between the majority cleaved PTPσ extracellular domains and the minority of uncleaved full-length proteins. PTPσ homophilic binding has only been assessed using systems in which full-length protein is rare (Haj et al., 1999). In these systems, as most of the PTPσ protein is soluble, even if individual molecules interact they will not be detected as binding to cells, therefore they show very little binding of PTPσ soluble extracellular domain to PTPσ expressing cells. Whilst these models replicate the in vivo situation it means that they cannot give a clear answer as to whether cleaved soluble and full-length PTPσ interact in trans. PTPσ has been shown above to form *cis*-dimers when expressed in the same cell, and some evidence exists that solublised PTPσ proteins can interact in solution (Figure 5.4). Therefore, the formation of *trans*-dimers by PTPσ expressed on different cells was assessed using two methods. Firstly, the method previously used by this laboratory was repeated using cells that expressed significant levels of uncleaved protein. In addition, PTPσ homophilic binding was assessed using the same techniques previously used to demonstrate homophilic adhesion in the related type IIb RPTPs.

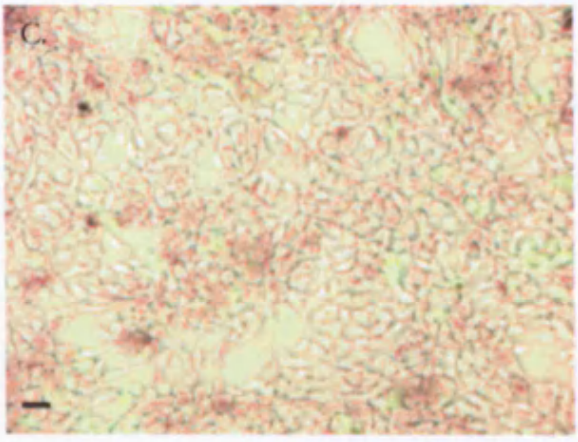
AP probe detection-Phase



GFP-Green

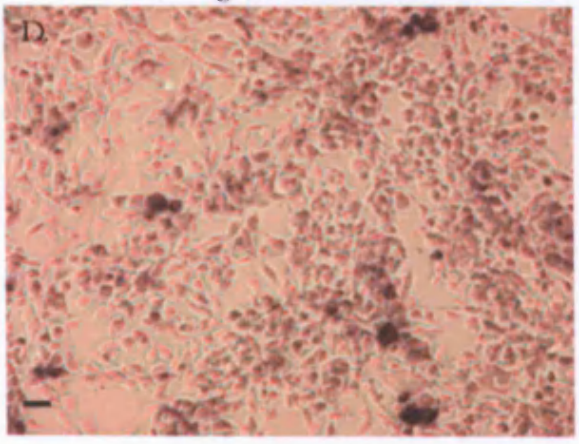


Merge

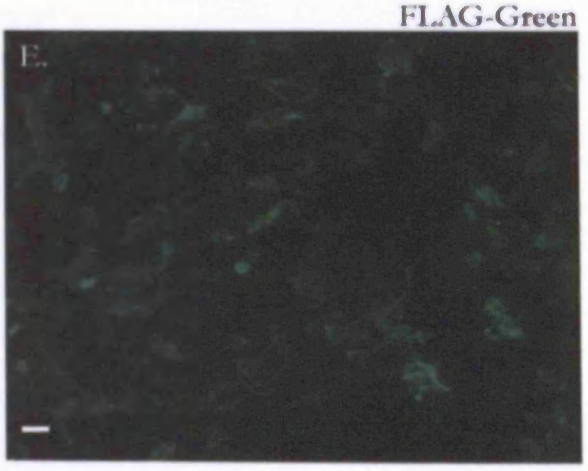


A-C:
Control transfection: GFP
Test probe: PTPσ-EC-AP

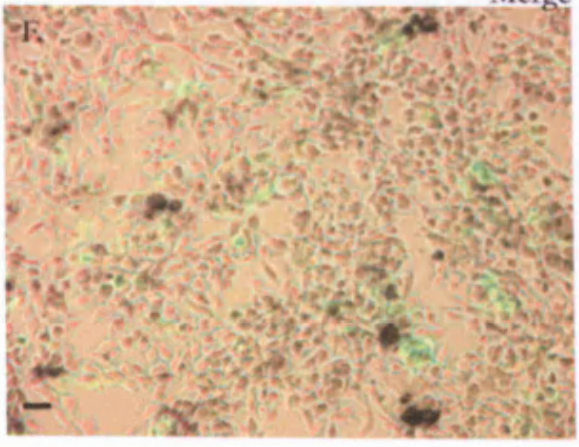
Background AP detection-Phase



FLAG-Green



Merge



D-F
Test transfection: 3xFLAG-PTPσ-HA
Control probe: GFP

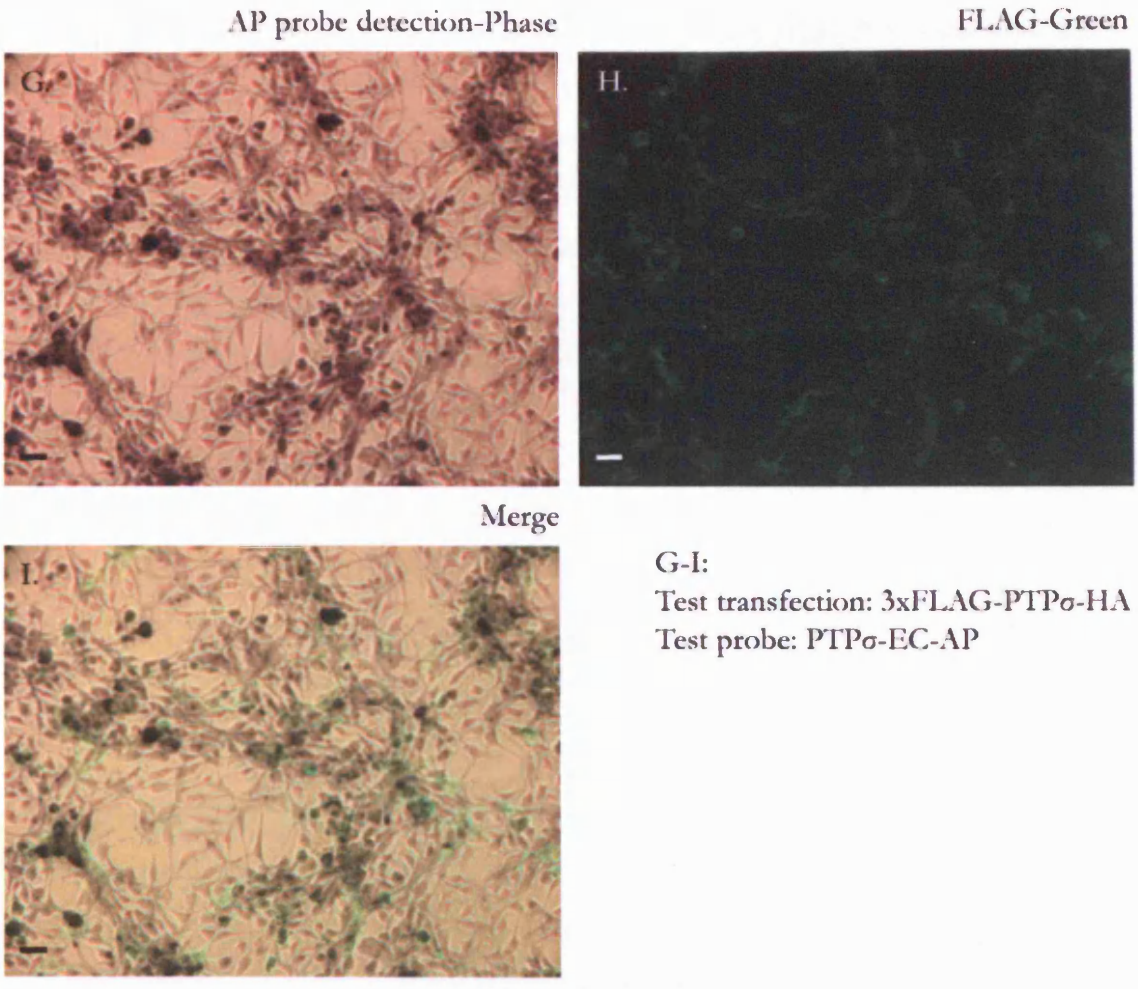


Figure 7.2: Analysis of homophilic binding with the RAP assay

HEK 293T cells were cultured on coverslips, transfected (A-C: Green fluorescent protein GFP; D-I: 3xFLAG-PTPσ-HA) and incubated with conditioned media (A-C. G-I: PTPσ-EC-AP; D-F: media from GFP-transfected cells). Coverslips were then washed, fixed and detected with anti-FLAG antibody and the AP colourigenic substrate NBT/BCIP. GFP was detected using its intrinsic epifluorescence in the FITC channel.

The control supernatant from cells expressing intracellular GFP lacked any detectable fluorescence in the GFP channel (Data not shown).

PTPσ-expressing cells may bind soluble PTPσ. When HEK-293T cells were heat-treated to inactivate cellular alkaline phosphatase, some background, endogenous cell-associated alkaline phosphatase signal remained (Figure 7.2 D). To avoid denaturation of possible PTPσ ligands, heat inactivation was carried out after incubation of cells with alkaline phosphatase (AP) probe and crosslinking of bound probe with parafomaldehyde. As the PTPσ-AP fusion exhibits resistance to heat inactivation, it is relatively unaffected by this treatment (Flanagan and Cheng, 2000; Flanagan et al., 2000). When cells expressing a control protein (GFP) were incubated with a PTPσ extracellular domain-alkaline fusion probe, there was very little increase in this background signal (Figure 7.2 A). However, when cells expressing PTPσ were incubated in conditioned media containing a soluble PTPσ extracellular domain-AP fusion, pilot experiments suggested that the proportion of cells showing AP activity increased. (Figure 7.2 G-I). However, a significant proportion of this increase in probe binding was in cells that did not themselves express PTPσ (Figure 7.2 C, F).

The RAP assay on PTPσ-transfected cells described above suggested that binding of soluble PTPσ extracellular domains is higher in populations of cells in which some express PTPσ than in cells that do not express significant levels of full-length protein. However, quantitative analysis of the effect was complicated by the requirements to normalise for transfection efficiency and background alkaline phosphatase activity. It remained unclear therefore whether PTPσ exhibits homophilic binding. This question was addressed using a technique previously validated for the related PTPμ (Cismasiu et al., 2004). Briefly, cells were incubated with soluble PTPσ extracellular domain in conditioned media, thoroughly washed and then processed for SDS-PAGE analysis. Bound PTPσ extracellular domain should only be detectable in the lysates if it has bound to the cells.

The PTPσ extracellular domain binds at a low level to the HEK-293T cell line and this binding is unaffected by transfection of HEK-293T cells with full-length PTPσ (Figure 7.3 C6-7). Soluble PTPσ extracellular domain-alkaline phosphatase fusions were produced in conditioned media as previously described (Figure 7.3 A, B1). Following incubation of control or PTPσ-transfected cells with conditioned media there was no detectable change in the level of PTPσ-AP fusion protein in the conditioned media (Figure 7.3 B1, B3-4). Similarly, although PTPσ-AP probe bound at low levels to HEK-293T cells, the amount of probe binding to HEK-293T cells was low and was unaffected by transfection with full-length PTPσ (Figure 7.3 C6-7). Together these suggest that quantitatively very little binding of PTPσ-AP probe to either control or PTPσ-transfected cells occurs. However, the rate of

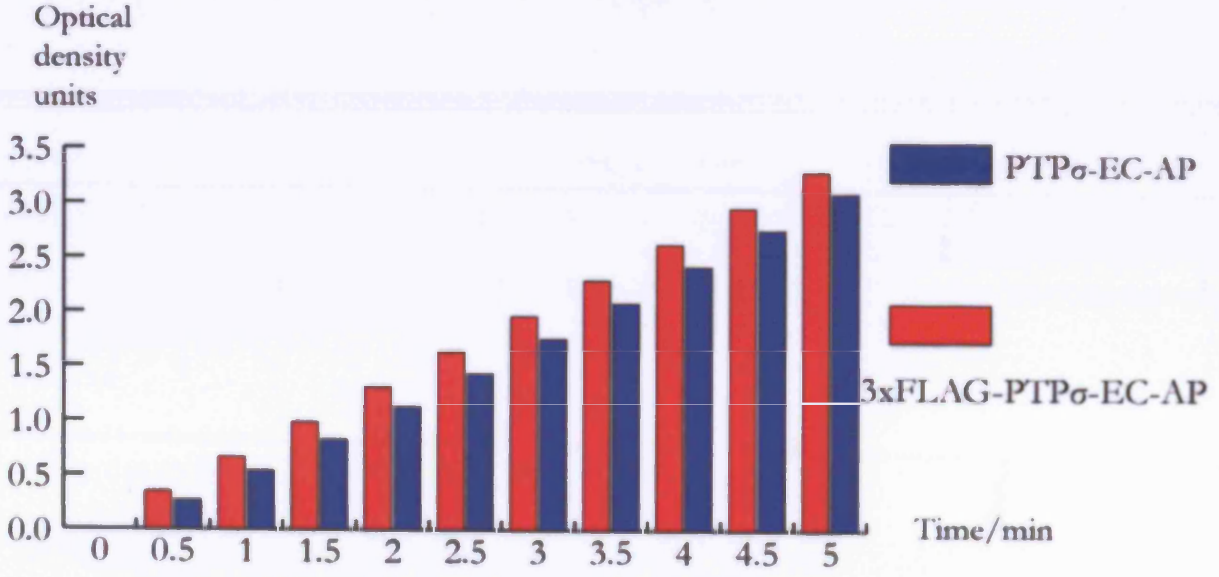
PTPσ extracellular domain shedding by full-length PTPσ-transfected HEK-293T cells was notable. Conditioned media containing the AP fusion proteins or the soluble extracellular domain cleavage fragment was collected 3 days after transfection. As previously described, this is the time point for maximal recovery of these fusion proteins (Aricescu, 2002). Similar amounts of soluble protein were found in both kinds of conditioned media at this time point. However, in media recollected after incubation with PTPσ-transfected cells for 8 hours, the amount of PTPσ shed by the cells was suprisingly high (Figure 7.3 B4).

7.1.3: His-tagged extracellular domain deletion series

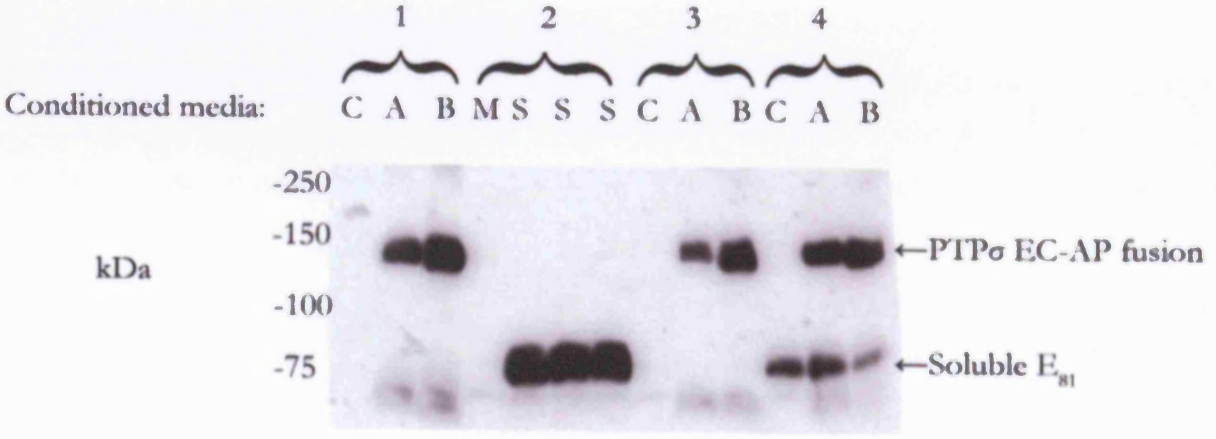
A series of PTPσ extracellular domain-alkaline phosphatase fusions were previously characterised and shown to affect the ability of PTPσ extracellular domains to bind the retinal HSPG ligand in a periodic manner (Chilton, 2000). However, the AP fusion constructs cannot be used to probe PTPσ dimerisation, as enzymatically active alkaline phosphatase is an obligate dimer. Therefore, a series of PTPσ ectodomain deletions were designed with His tags replacing the AP moiety to allow large-scale purification of the PTPσ extracellular domains (pHL-PTPσ-FNΔ3 provided by Dr R. Aricescu). These constructs were designed to assist the identification of PTPσ ligands and for subsequent use in biochemical experiments. These experiments will include dynamic light scattering and crystallographic approaches to determine the structure of PTPσ and characterise the minimum domain requirements for PTPσ dimerisation.

Expression and secretion of enzymatically active protein (where appropriate) was confirmed by SDS-PAGE analysis and phosphatase activity assay using medium conditioned by cells transfected with the His-tagged PTPσ constructs. All of the recombinant proteins were expressed at their expected molecular weights (SAP.His – His-tagged soluble alkaline phosphatase (56.1 kDa – data not shown), PTPσ-FNΔ2.His (55.8 kDa), PTPσ-FNΔ3.His (66.3 kDa), PTPσ-FNΔ4.His (77.5 kDa), PTPσ-FNΔ3-AP.His (120.6 kDa)) by transfected HEK-293T cells (Figure 7.4 A, B, C).

A. Alkaline phosphatase activity of the PTPσ-AP fusion proteins



B. Conditioned media



C. Lysates

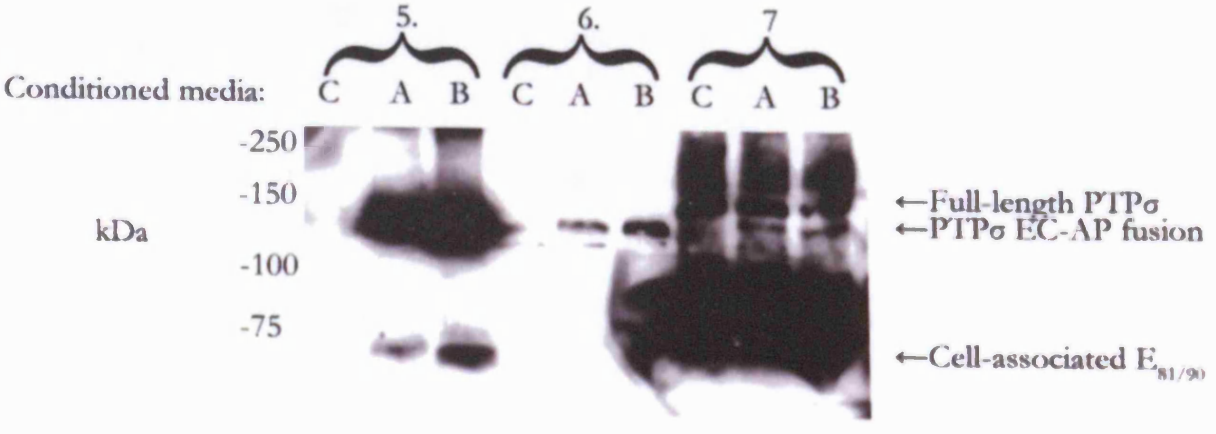


Figure 7.3: Analysis of homophilic binding by SDS-PAGE

A. The phosphatase activities of FLAG tagged and untagged PTPσ extracellular domain-alkaline phosphatase fusions were compared using a colourigenic substrate (*p*-Nitrophenylphosphate (pNPP)).

Conditioned media was harvested after three days from cells expressing FLAG-tagged (A) or untagged (B) PTPσ-AP probes or GFP (C). Media was passed through a 0.22μm filter to remove cells and debris and analysed by SDS-PAGE. In addition, cells transfected with GFP- or full length PTPσ were incubated with this conditioned media for eight hours. After eight hours, the media was re-collected and the cells were washed and lysed for SDS-PAGE analysis as before. PTPσ proteins were detected with the IG2 antibody.

B. Conditioned media before and after incubation with cells expressing GFP or full length PTPσ.

1. Conditioned media collected after three days from cells expressing PTPσ-AP probes (A – FLAG-tagged; B – untagged) or GFP (C).
2. Conditioned media collected after 3 days from mock-transfected (M) or PTPσ-expressing (S) cells.
3. Conditioned media from 1 (above) recollected after an 8 hour incubation with GFP-expressing cells.
4. Conditioned media from 1 (above) recollected after an 8 hour incubation with PTPσ-expressing cells.

C. Lysates from cells expressing GFP or full length PTPσ after incubation with conditoned media.

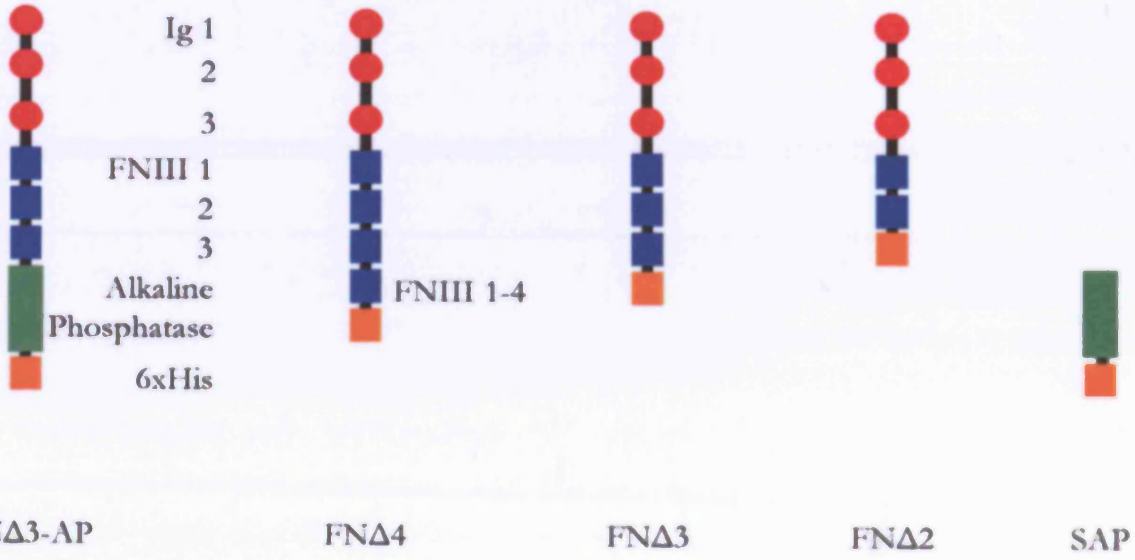
5. Conditioned media as shown in B1 (above).
6. Lysates from GFP-expressing cells after an 8 hour incubation with control (C) or PTPσ-AP containing conditioned media (FLAG-tagged (A/B as above)
7. Lysates from full length PTPσ-expressing cells after an 8 hour incubation with control (C) or PTPσ-AP containing conditioned media (A/B as above).

PTPσ-His tagged proteins form higher order oligomers in solution (Figure 7.4 B). In addition to the expected bands, lysates from FNΔ2.His-, FNΔ3.His-, FNΔ4.His- and FNΔ3AP.His-transfected cells showed small amounts of a higher molecular weight protein. In FNΔ2.His- and FNΔ3.His-transfected lysates, this protein ran at about 150 kDa and 180 kDa respectively, commensurate with trimers of these two proteins. In contrast, FNΔ4.His- and FNΔ3AP.His transfected cells migrate at 150 kDa and >200 kDa respectively, commensurate with dimers of these two proteins. The FNΔ3AP.His protein was expected to form dimers (~240kDa) due to its incorporation of the AP moiety, which is an obligate dimer. However, the nature of these species in the other samples was not clear. Although higher molecular weight species were not detected from cells expressing the original FNΔ3AP protein from the series designed by John Chilton, this protein expressed at much lower levels than the His-tagged proteins (Figure 7.4 B).

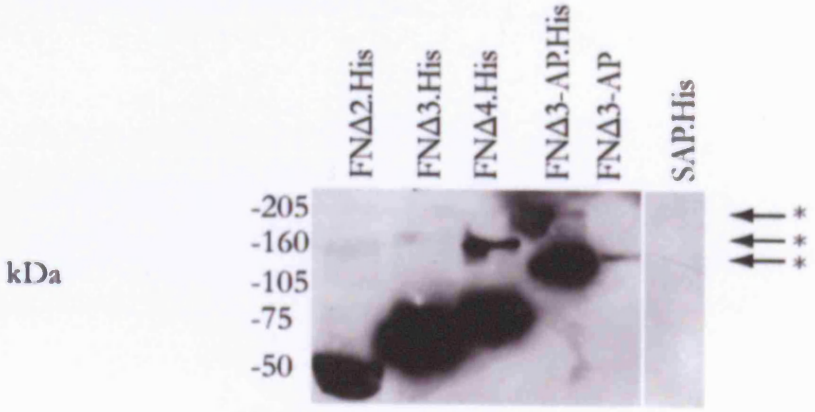
7.2: Intracellular domain interactions

Whilst it has been difficult to identify ligands for RPTPs, it has also been difficult to identify proteins that interact with the intracellular domain including any candidate phosphotyrosine substrates. Cysteine-based PTPs exhibit high basal levels of activity and show broad substrate specificity *in vitro* (Zhang, 2002). Therefore, it is difficult to determine whether proteins identified in vitro as substrates are physiological substrates or merely artefacts of the cell type or expression system used. To date, there are no known PTPσ substrates and the only known interacting partners for the PTPσ intracellular domain are the intracellular domains of RPTPs including those of PTPσ itself (Wallace et al., 1998; Blanchetot and den Hertog, 2000b; Blanchetot et al., 2002b). To characterise the intracellular domain interactions of PTPσ, the co-immunoprecipitation method that was used with good effect above to show PTPσ dimerisation was used to co-immunoprecipitate proteins that interact with PTPσ.

A.


Ig 1
2
3
FNIII 1
2
3
Alkaline
Phosphatase
6xHis
FNIII 1-4
FNΔ3-AP
FNΔ4
FNΔ3
FNΔ2
SAP

B.


FNΔ2.His
FNΔ3.His
FNΔ4.His
FNΔ3-AP.His
FNΔ3-AP
SAP.His
kDa
-205
-160
-105
-75
-50
*
*
*

C.

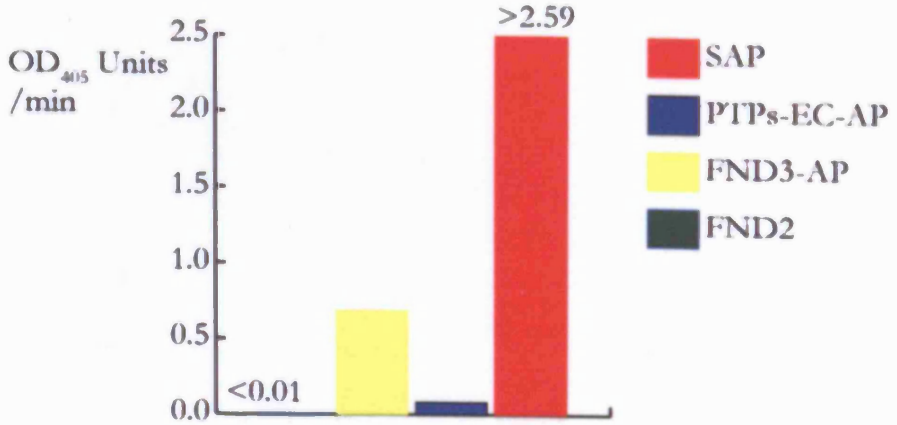

OD405 Units /min
2.5
2.0
1.5
1.0
0.5
0.0
<0.01
>2.59
SAP
PTPs-EC-AP
FND3-AP
FND2

Figure 7.4: Expression of soluble 6xHis-tagged PTPσ extracellular domain deletion proteins

A. Schematic diagram of PTPσ extracellular domain deletion proteins showing their domain organisation and location of 6xHis and alkaline phosphatase moieties.
B. Reducing SDS-PAGE analysis of conditioned media from HEK-293T cells transfected with the indicated PTPσ extracellular domain deletions or soluble alkaline phosphatase. * - High molecular weight species
C. Graph showing alkaline phosphatase activity of conditioned media from HEK-293T cells transfected with the indicated PTPσ extracellular domain deletions or soluble alkaline phosphatase.

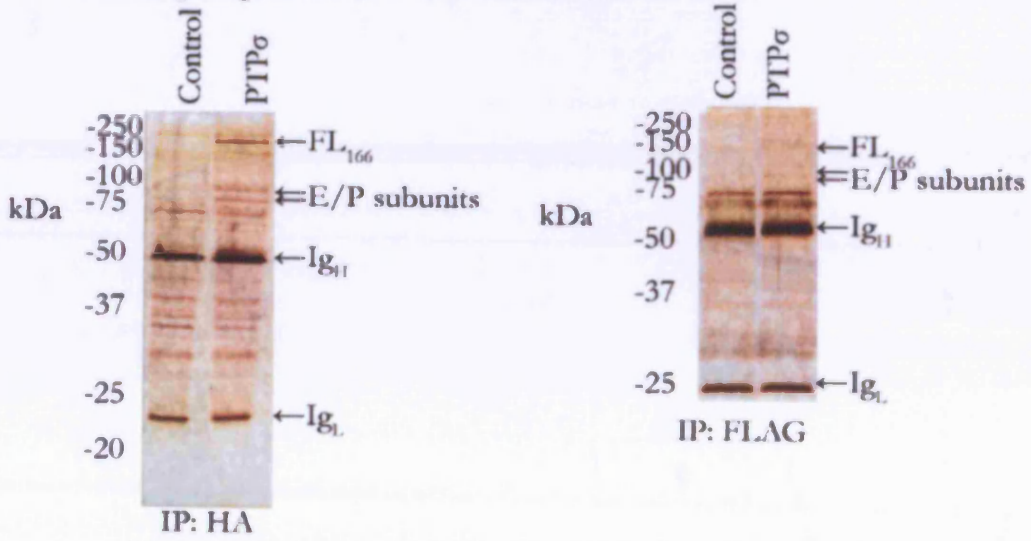
7.2.1: Co-immunoprecipitation of wild-type PTPσ

Co-immunoprecipitation experiments using full-length wild type PTPσ failed to isolate any identifiable PTPσ binding proteins. Robust levels of PTPσ expression were obtained and following immunoprecipitation, purified PTPσ could be identified on SDS-PAGE gels detected with silver or brilliant blue stains to show total protein (Figure 7.5 A). However, it was not possible to identify any other co-purifying bands due to the high background level of bands that co-immunoprecipitated with the anti-FLAG or anti-HA antibodies under these conditions. Immunoblot analysis of immunoprecipitates confirmed robust immunoprecipitation of PTPσ. Moreover, both extracellular domain cleavage fragments ($E_{90/81}$) immunoprecipitated with antibodies directed against the carboxy-terminal tag and both intracellular domain cleavage fragments ($P_{85/75}$) immunoprecipitated with antibodies directed against the amino-terminal tag (Figure 7.5 C, D). Significantly, more full-length than cleaved protein immunoprecipitated in these experiments.

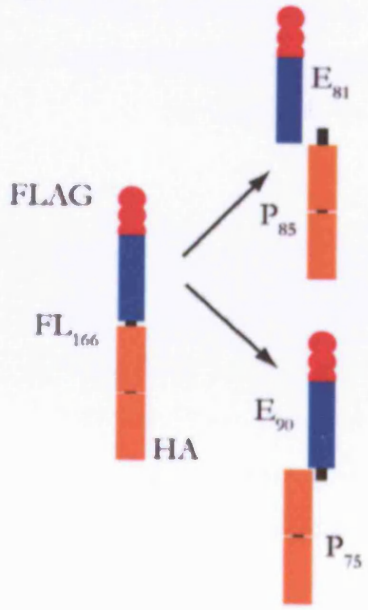
7.2.2: Co-immunoprecipitation of substrate trapping mutant PTPσ

The aim of the immunoprecipitation experiments described above was first and foremost to identify PTPσ substrates and only then to investigate other PTPσ-interacting proteins. However, many protein-protein interactions are transient, occurring only for short periods of time or only under specific conditions which may themselves only be of brief duration. This is especially likely to be true of tyrosine phosphatase-substrate interactions. The tyrosine phosphate group is one of the major determinants of substrate binding. A part of the tyrosine phosphatase catalytic mechanism, this phosphate group is transferred to an enzyme thio-phosphate intermediate. As tyrosine phosphatases exhibit high levels of activity at rest, a major part of the substrate binding mechanism is likely to be lost soon after substrate binding (Streuli et al., 1989; Guan et al., 1990). This is likely to make substrate-enzyme interactions very brief.

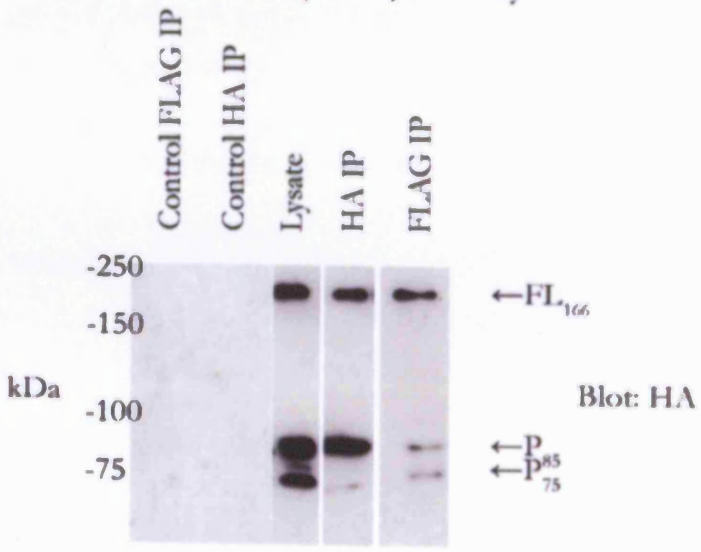
A. Immunoprecipitation of PTPσ - Silver stain



B.



C. Immunoprecipitation of $P_{85/75}$ subunits with extracellular domain (FLAG) antibody



D. Immunoprecipitation of $E_{90/81}$ subunits with intracellular domain antibody

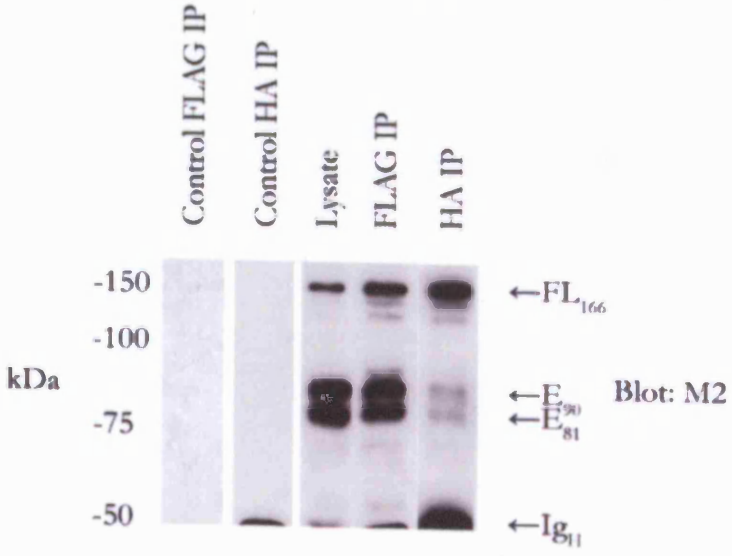


Figure 7.5: Immunoprecipitation of wild-type full-length PTPσ

A. Immunoprecipitation of PTPσ using antibodies directed against the extracellular FLAG or intracellular HA tag. Immunoprecipitates were analysed under reducing conditions using SDS-PAGE (12.5% total acrylamide gel). Gels were stained for total protein using a non-ammoniacal silver stain.

B. Schematic diagram of PTPσ cleavage products showing epitope tag locations

C. Immunoprecipitation of $P_{85/75}$ subunits with antibody directed against the extracellular domain FLAG tag.

D. Immunoprecipitation of $E_{90/81}$ subunits with antibody directed against the intracellular domain HA tag.

PTPσ and immunoglobulin species are labelled. Control immunoprecipitates were carried out on lysates from cells expressing a non-HA/FLAG tagged form of PTPσ.

Cellular phosphotyrosine levels are maintained at relatively low levels compared for example to serine or threonine phosphate due to the tightly regulated activity of tyrosine kinases and the high basal activity level of cellular tyrosine phosphatases (Hunter and Sefton, 1980; Streuli et al., 1989; Guan et al., 1990). Several strategies exist to boost cellular phosphotyrosine levels to increase the likelihood of the phosphatase substrate being present in its phosphorylated form at a sufficient level for purification and subsequent identification. Briefly, cells may be treated with substances that increase tyrosine kinase activity or they may be treated with tyrosine phosphatase inhibitors. Stimulants of tyrosine kinase activity are generally pathway specific agents such as growth factors whose use results in significant increases in cellular phosphotyrosine level that are concentrated in a small number of proteins. The choice of a suitable agent therefore requires some degree of thought and a measure of luck if it is to stimulate the phosphorylation of the appropriate substrate proteins. On the other hand, most tyrosine phosphatase inhibitors are not targeted but inhibit all cellular tyrosine phosphatases, resulting in significant increases in cellular phosphotyrosine spread across a large range of proteins. This effect is believed to be due both to the inhibition of substrate dephosphorylation and to the inactivation of those phosphatases that are responsible for dephosphorylating the activation loop tyrosine in tyrosine kinases, so increasing kinase activity in a general manner at the same time as decreasing phosphatase activity in a pan-cellular manner.

Tyrosine phosphatase enzymes can be altered by site-directed mutagenesis to forms that bind substrate but are unable to catalyse hydrolysis of the tyrosine phosphatase bond. Such mutations are known as "substrate-trapping" mutations or simply as trapping mutations. A number of tyrosine phosphatase trapping mutations exist (Blanchetot et al., 2005). These mutations may be divided into mutations that perturb the catalytic cysteine, mutations that affect the WPD loop and mutations that affect the chelation of an essential water molecule required for phosphatase function. The characteristics of these mutants and the theoretical differences in the effects of tyrosine phosphatase inhibitors on wild type and trapping mutant enzymes were described above (1.1.3).

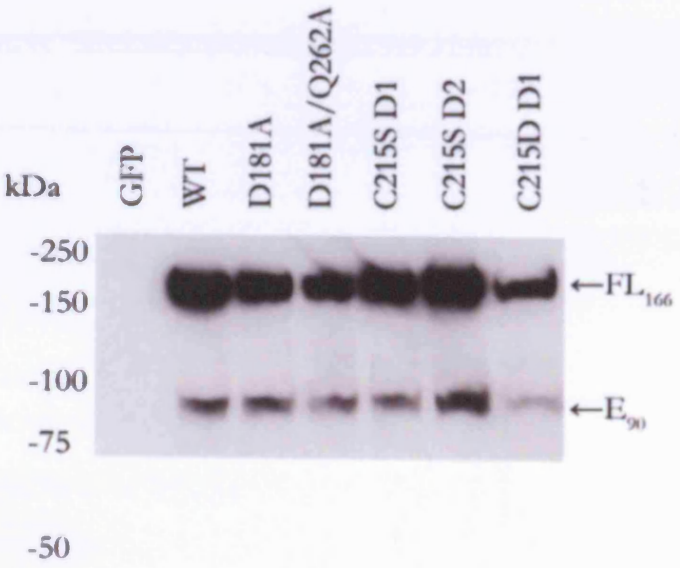
Expression of wild type or substrate trapping PTPσ did not alter the cellular phosphotyrosine level. Wild type PTPσ and PTPσ containing each of four D1 trapping mutations and one D2 trapping mutation were expressed in HEK-293T cells (Figure 7.6A). As previously described, phosphotyrosine levels in HEK-293T cells were undetectable in the absence of a phosphotyrosine inhibitor. Following phosphatase inhibitor (pervanadate) treatment although phosphotyrosine levels were detectable, no significant difference could

be detected between control cells and cells transfected with wild type or substrate trapping PTPσ (Figure 7.6B).

Immunoprecipitation of substrate trapping PTPσ detected several phosphotyrosine positive putative substrates but did not allow their identification. Immunoprecipitation of wild type or substrate trapping PTPσ in the absence of tyrosine phosphatase inhibition failed to immunoprecipitate any tyrosine phosphorylated bands. However, following pervanadate treatment, immunoprecipitates from both wild type and substrate trapping PTPσ contained three major phosphotyrosine positive bands. These species corresponded to the expected size of full-length PTPσ and the PTPσ intracellular domain cleavage fragments (Figure 7.7A, B). It is notable that the phosphotyrosine content of these bands was significantly higher in immunoprecipitates from substrate trapping PTPσ-expressing cells than in immunoprecipitates from wild type PTPσ-expressing cells. As previously described above (6.2), the intracellular cleavage fragments of both wild type and now substrate trapping PTPσ were seen to exhibit higher levels of tyrosine phosphorylation than full-length protein. In addition to these PTPσ bands, small amounts of tyrosine phosphorylated protein could also be seen in some of the samples containing substrate trapping PTPσ that migrated at approximately 40 kilodaltons (D181A) and at approximately 60 kilodaltons (D181A, C215S D1) (Figure 7.7A). A band that immunoprecipitates with the D181A trapping mutations has previously been detected but not identified (Aricescu, 2002). Several other faint bands could be seen ranging from 25 to 50 kilodaltons; note the generally increased signal density and the presence of some specific bands in the 25-50kDa region (Figure 7.7A lane 5-8 when compared to lanes 1-4).

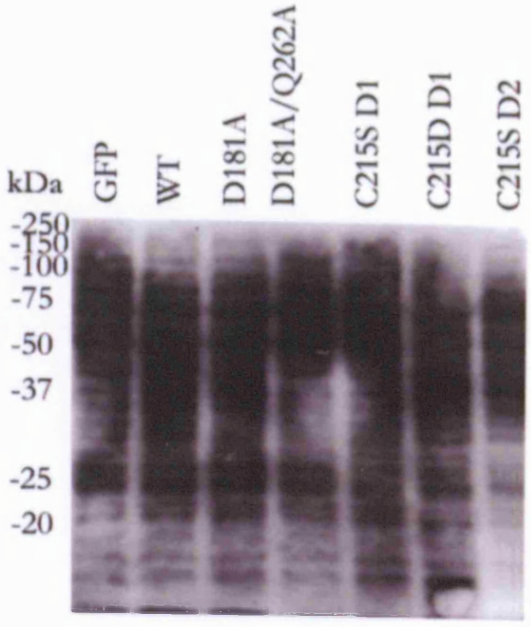
Analysis of these gels using a non-ammoniacal silver stain to detect total protein revealed a very large number of proteins in both control and PTPσ immunoprecipitates (Figure 7.8A, B). This hindered the identification of the phosphotyrosine positive bands and their subsequent excision and identification by mass spectrometry. Although a 40 kilodalton species can be identified in pervanadate treated D181A/Q262A and C215D D1 immunoprecipitates (Figure 7.8B lanes 1, 3), it did not prove possible to excise sufficient amounts of pure protein to identify this species. In addition, small amounts of this peptide could be seen in C215S D2 immunoprecipitates (Figure 7.8B lane 5).

A. Expression of Trapping mutant PTPσ



Blot: Anti-FLAG(M2)

B. Phosphotyrosine content of control and PTPσ expressing lysates



Blot: Anti-phosphotyrosine (4G10)

Figure 7.6: Expression of "substrate trapping" PTPσ proteins

HEK 293T cells were transfected with expression vectors containing either GFP or PTPσ (Wild type, D181A, C215S D1, D181A/Q262A, C215D D1 or C215S D2)

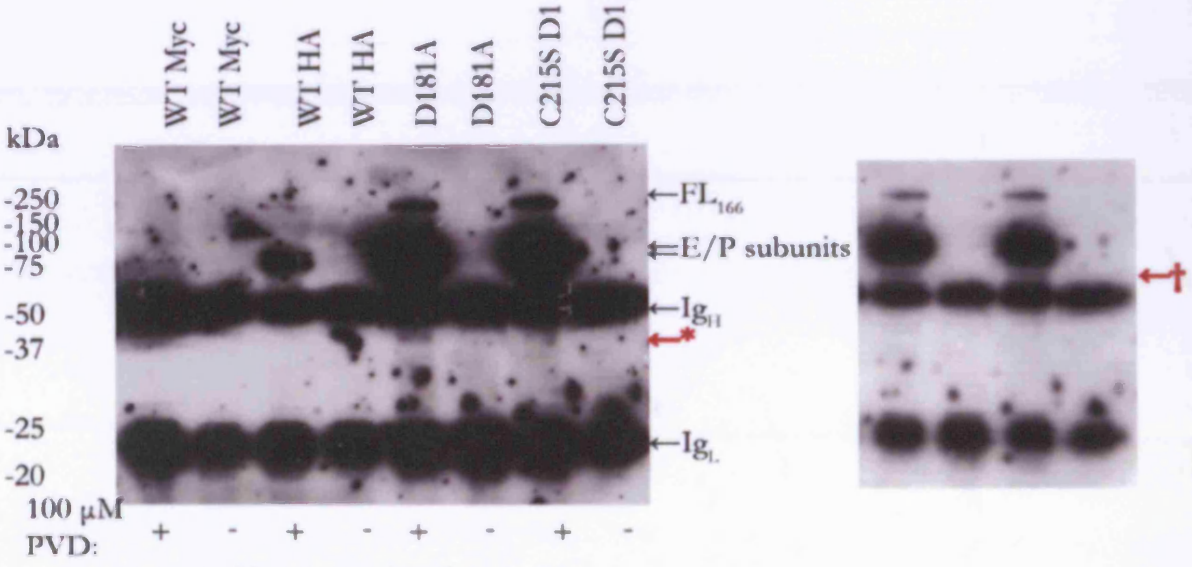
A. Expression of PTPσ wild type and substrate trapping proteins

Cells were lysed and analysed by SDS-PAGE. Western blots were detected with an antibody (M2) directed against the amino-terminal FLAG tag.

B. Phosphotyrosine content of cells transfected with control protein (GFP), wild type PTPσ or substrate trapping PTPσ.

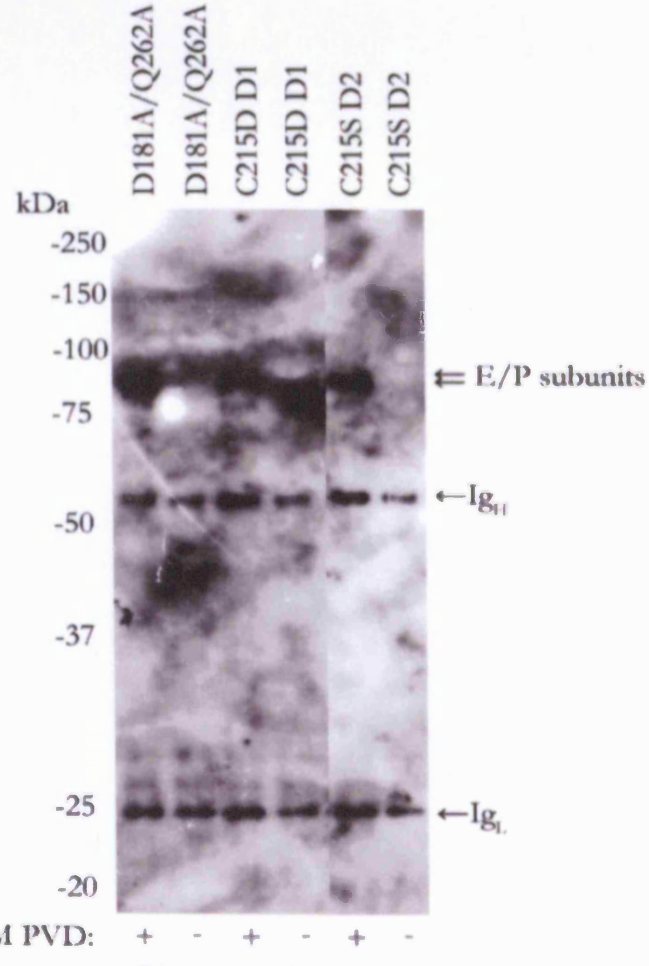
Following incubation with pervanadate, cells were lysed and analysed by SDS-PAGE. Western blots were detected with the 4G10 anti-phosphotyrosine antibody.

A. Phosphotyrosine content of substrate trapping PTPσ immunoprecipitates (WT, D181A, C215S D1)



Blot: Anti-phosphotyrosine

B. Phosphotyrosine content of substrate trapping PTPσ immunoprecipitates (D181A/Q262, C215D D1, C215S D2)



Blot: Anti-phosphotyrosine

Figure 7.7: Immunoprecipitation of substrate trapping PTPσ 1 – Phosphotyrosine detection

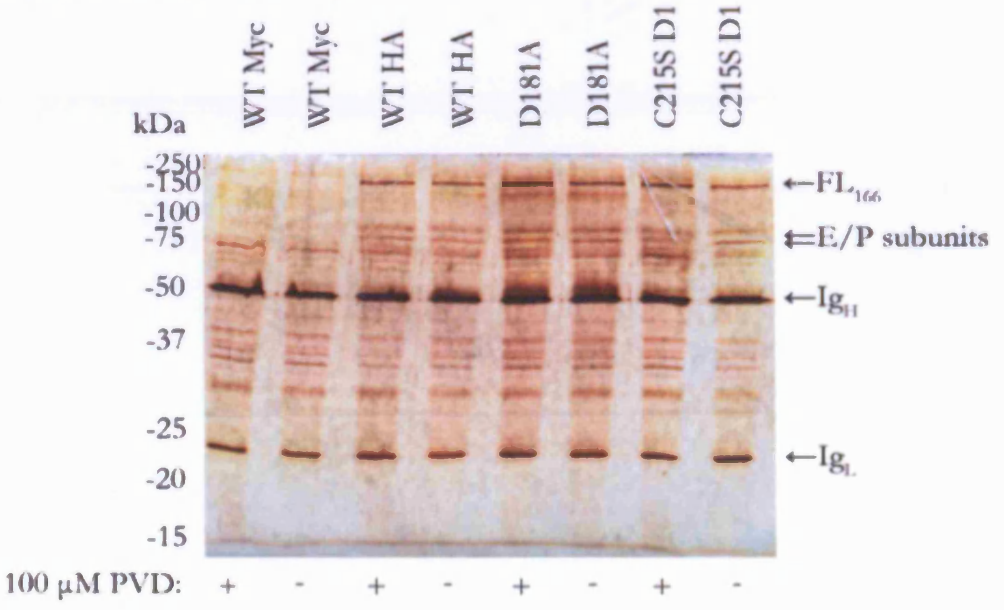
A. Anti-HA immunoprecipitates from WT-Myc, WT-HA , D181A-HA and C215S D1-HA expressing HEK-293T cells

Smaller panel shows shorter exposure of lanes 5-8. Red asterisk (*) indicates phosphotyrosine positive putative substrate (Mw~ 40kDa) in pervanadate treated D181A sample. Red dagger (†) indicates phosphotyrosine positive putative substrate (Mw~60kDa) in pervanadate treated D181A and C215S D1 samples.

B. Anti-HA immunoprecipitates from D181A/Q262A-HA, C215D D1-HA and C215S D2-HA expressing HEK-293T cells.

Treatment with or without 10μM pervanadate is indicated below each major figure. PTPσ full-length protein (FL_{166}) and PTPσ cleavage fragments (E/P subunits) are indicated. Antibody heavy (Ig_H, Mw~50kDa) and light (Ig_L, Mw~25kDa) bands are marked. In lane 4, the small amount of phosphotyrosine-positive PTPσ E/P subunits at 80-90kDa is partially masked by a defect on the gel.

A. Peptide content of substrate trapping PTPσ immunoprecipitates
(WT., D181A, C215S D1)



B. Peptide content of substrate trapping PTPσ immunoprecipitates
(D181A/Q262A, C215D D1, C215S D2)

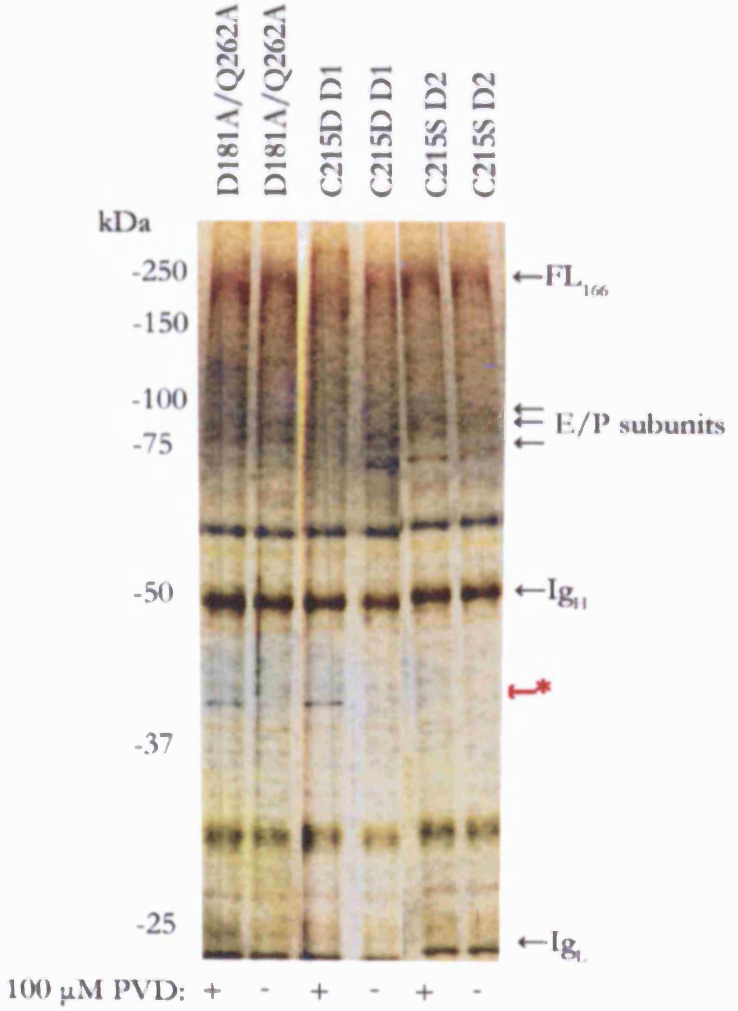


Figure 7.8: Immunoprecipitation of substrate trapping PTPσ 2 – Silver stain

A. Anti-HA immunoprecipitates from WT-Myc, WT-HA , D181A-HA and C215S D1-HA expressing HEK-293T cells

B. Anti-HA immunoprecipitates from D181A/Q262A-HA, C215D D1-HA and C215S D2-HA expressing HEK-293T cells.

Treatment with or without 10μM pervanadate is indicated below each major figure. The size of the PTPσ full-length protein (FL_{166}) and PTPσ cleavage fragments (E/P subunits) are indicated. Antibody heavy (Ig_H, Mw~50kDa) and light (Ig_L, Mw~25kDa) bands are marked.

Red asterisk (*) indicates putative PTPσ interacting protein (Mw~ 40kDa) in pervanadate treated D181A/Q262A, C215D D1 and C215S D2 samples.

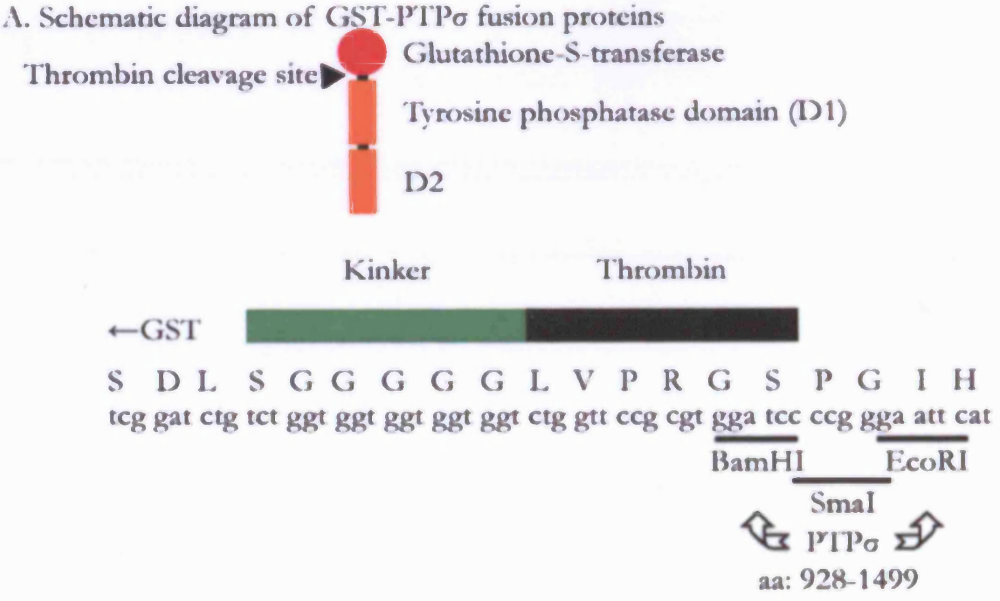
7.2.3: Construction of GST-PTPσ fusion proteins

Co-immunoprecipitation of substrate trapping mutant PTPσ did not yield sufficient amounts of the putative substrate-trapped interacting proteins to allow their identification. It was important therefore to increase the scale and stringency of the immunoprecipitations. A range of alternative methods of high-level recombinant protein production such as yeast and bacterial expression systems exist together with several large-scale purification techniques. One of the most widely used systems, which combines both high-level recombinant protein production and easy purification is the glutathione-S-transferase pull-down technique. This commonly uses elements from the *lac* operon to allow the induction of recombinant protein expression by addition of a lactose analogue (e.g. Isopropyl-1-thio-β-D-galactoside – IPTG) to the culture medium. The recombinant protein is commonly fused with a GST moiety. GST binds strongly to glutathione and glutathione immobilised on beads can be used to purify the protein of interest from induced bacterial cell lysates. This results in columns of immobilised GST -PTPσ protein, down which lysates from mammalian cells can be passed. Several PTPσ constructs were generated that allowed the inducible expression of wild type and substrate trapping (D181A, Q262A, Dbl, C215S, C215D) PTPσ. These constructs contained the entire PTPσ intracellular domain (bp: 2743-4701; a.a.: 928-1499) located behind a single GST moiety (Figure 7.9 A). The expected molecular weights of GST and the GST-PTPσ fusions are ~27.5 kDA and ~98.4kDa, respectively.

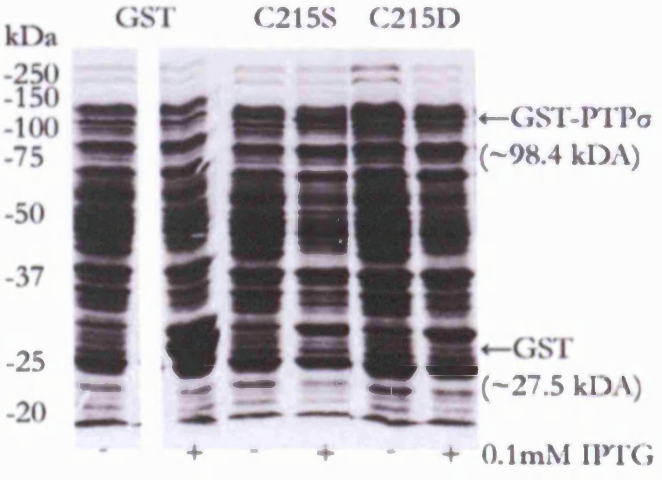
It did not prove possible to derive conditions allowing the expression of all of the GST-PTPσ fusion proteins. Evaluation of the GST-PTPσ fusion proteins was carried out in collaboration with Dr Clare Faux (Neural Development unit, ICH). It was only possible to obtain expression of two of the GST-PTPσ fusion proteins, those containing the C215S and C215D mutations. In the absence of IPTG, only low-level "leaky" expression of GST, GST-PTPσ C215S D1 and GST-PTPσ C215D D1 fusion protein was detected (Figure 7.9 D). In contrast, following incubation with inducing concentrations of IPTG, robust expression of the GST, GST-PTPσ C215S D1 and GST-PTPσ C215D D1 fusion proteins was detected (Figure 7.9 B). Indeed expression levels were high enough to allow the GST and GST-PTPσ fusion proteins to be identified on SDS-PAGE gels using Brilliant Blue staining (Figure 7.9 B). Although significant amounts of the C215S GST-PTPσ fusion protein were produced following induction at 37°C, at this temperature the C215D GST fusion protein was insoluble. Soluble GST-PTPσ C215D expression was only possible following induction at 25-28°C. It did not prove possible to derive suitable conditions for

the expression of the wild type, D181A or D181A/Q262A GST-PTPσ fusion proteins (Dr Clare Faux; Personal communication). Further analysis of these fusion proteins and their potential use to "pull-down" PTPσ interacting proteins is being continued by Dr Clare Faux.

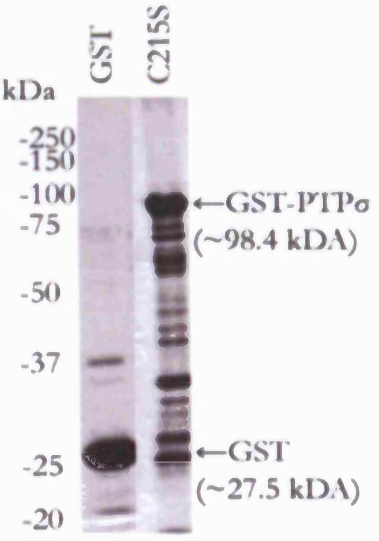
A. Schematic diagram of GST-PTPσ fusion proteins



B. GST-PTPσ fusion protein expression 1:
Brilliant Blue stain



C. GST fusion protein column purification
Brilliant Blue stain



Brilliant blue stain

D. GST-PTPσ fusion protein expression 2:
Immunoblot: Anti-GST detection

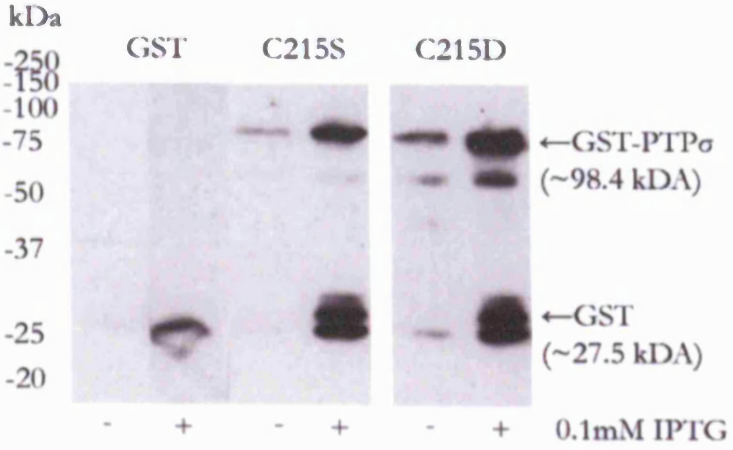


Figure 7.9: Expression of PTPσ-GST fusion proteins

A. Schematic diagram of PTPσ-GST fusion proteins

The PTPσ intracellular domain (a.a. 928-1499) was cloned into the pGEX-KT vector between the *BamHI* and *EcoRI* sites, 3' to the glutathione-S-transferase (GST) moiety.

B. GST-PTPσ fusion protein expression 1: Brilliant Blue stain

D. GST-PTPσ fusion protein expression 2: Anti-GST detection

Bacterial cells were transformed with the appropriate GST expression vector and lysed with or without induction for 4 hours with 0.1mM Isopropyl-1-thio-β-D-galactoside (IPTG). Lysates were analysed using SDS-PAGE. Gels were stained using Brilliant Blue G-250 (B) or blotted to PVDF membranes and detected using an anti-GST antibody (D). GST alone, GST-PTPσc215S and GST-PTPσ215D are shown.

C. GST fusion protein column purification. Brilliant Blue stain

Glutathione-bead purification of GST and GST-PTPσ C215S fusion protein. Column purification was kindly carried out by Dr Clare Faux (Neural Development Unit, ICH). Bacterial cell lysates were processed using glutathione beads and the resulting eluates concentrated as described above. Concentrated protein samples produce in this manner were analysed by SDS-PAGE. Gels were stained with Brilliant Blue G-250.

The expected sizes of the GST (27.5 kDa) and GST-PTPσ fusions (98.4 kDa) are indicated to the right of the gels.

7.3: Discussion

This chapter describes attempts to identify PTPσ-interacting proteins. Unfortunately, it was not possible to identify any proteins that interacted with PTPσ; however, a number of experimental tools were produced that may in the future increase our understanding of PTPσ function. The approaches used may be divided into those that looked at the interactions of the PTPσ extracellular domain and those that looked at the interactions of the intracellular domain.

PTPσ ligands were located using fusions of the PTPσ extracellular domain with alkaline phosphatase (AP) (Haj et al., 1999; Sajnani-Perez et al., 2003). However, only dimeric AP is enzymatically active (Holmgren et al., 1977). Therefore, it is not yet known whether the orientation of the PTPσ-AP fusions is determined by the AP or the PTPσ components, which has obvious significance for the authenticity of ligands identified in this manner. However, only dimeric PTPσ extracellular domains recognise the basement membrane ligand (Lee et al., 2006a). Although fusion of the AP moiety with PTPσ did not interfere with PTPσ ligand recognition, this may depend on the site of fusion (Chilton, 2000). Such a phenomenon might explain the difficulties many researchers have had using fusions of RPTPs other than PTPσ with AP. The PTPσ-AP fusions may have been serendipitously designed such that they form ligand-binding sites whereas other researchers may not have had the same luck when they designed their AP fusion proteins. The basement membrane ligands bound by the PTPσ-AP probes are also recognised by a VSV-tagged PTPσ extracellular domain probe, which would not suffer from the same structural constraints (Rashid-Doubell et al., 2002). However, although VSV-tagged PTPσ was readily detectable in solid phase binding assays, it proved difficult to detect following long term *in vivo* expression (Aricescu et al., 2002; Rashid-Doubell et al., 2002). This might reflect the expression level of the probe or the accessibility of the tag. In order to further optimise tag detection, the 3xFLAG tag was used, which is larger, containing three repeats of the tag sequence, and more polar than many other epitope tags.

The addition of a 3xFLAG tag to the extreme amino-terminus of the mature PTPσ protein did not interfere with ligand binding (Figure 7.1) (Aricescu et al., 2002; Sajnani-Perez et al., 2003). These data suggest that tags located at the extreme amino-terminal end of the PTPσ protein have only minimal effects on the binding of known PTPσ ligands and are a useful adjunct to AP fusion proteins for identifying and characterising PTPσ ligands. In particular, analysis of the binding pattern of epitope-tagged PTPσ extracellular domain

probes that lack the AP moiety will determine several important questions. As it has recently been shown that only dimeric PTPσ is able to bind basement membrane ligands, if epitope-tagged PTPσ extracellular domains also bind ligand then it may be concluded that PTPσ extracellular domains form dimers in solution (Lee et al., 2006a). Unfortunately, it may continue to prove difficult to detect these dimers directly. Secondly, if the hypothesis that the binding properties of truncated PTPσ-AP fusions are due to the constraining effect of the AP dimer interface is correct, then ligand binding assays using epitope-tagged PTPσ extracellular domains and no AP component might fail to detect a difference in binding specificity between FNΔ5 and FNΔ6, for example. Importantly, this experiment would also determine whether there is a real difference in ligand binding between the two major PTPσ isoforms, PTPσ1 and PTPσ2.

In addition, epitope tagged PTPσ was used to reassess whether PTPσ exhibits homophilic binding using cells expressing high levels of full length PTPσ at the cell membrane. This thesis has previously shown that PTPσ forms dimers on the cell surface. However, it was not possible to determine whether these dimers represented the interaction of PTPσ molecules expressed on the same cell (in *cis*) or the interaction of PTPσ molecules expressed on neighbouring cells (in *trans*). *Trans*-interactions of this kind are known as homophilic adhesion and are a common feature of many adhesion molecules including several other RPTPs (Brady-Kalnay et al., 1993; Gebbink et al., 1993b; Brady-Kalnay and Tonks, 1994; Sap et al., 1994; Zondag et al., 1995; Wang and Bixby, 1999; Baker et al., 2000b; Yang et al., 2003; Cismasiu et al., 2004; Yang et al., 2005; Aricescu et al., 2006). However, previous work has found little evidence that PTPσ exhibits homophilic binding. This may be related to the absence of the LASE-c/meC alternatively spliced exons, which may be important for PTPδ/LAR homophilic adhesion, in the PTPσ proteins assayed (O'Grady et al., 1994; Pulido et al., 1995a; Yang et al., 2003; Yang et al., 2005). Indeed, careful examination of previous studies might suggest that PTPσ-AP probe binding decreases when cells are transfected with PTPσ (Haj et al., 1999). In this thesis, a PTPσ-AP probe was shown to bind to HEK-293T cells and binding was seen to increase following transfection of the HEK-293T cells with full-length PTPσ. Interestingly, PTPσ-AP probe binding appeared to increase following transfection with PTPσ not only in PTPσ-expressing cells but also in neighbouring cells. This may be related to the recent finding that PTPσ can act as a ligand as well as a receptor (Sajnani et al., 2005) and suggests that a possible candidate for the PTPσ ligand present on HEK-293T cells might be bound PTPσ extracellular domain shed by the transfected cells. Unfortunately, if this pattern of binding

both to cells transfected with PTPσ and to neighbouring untransfected cells is correct, this may make the detection and interpretation of probe binding significantly more difficult to investigate using conventional methods. Probe binding would then require transfection of the cell population with PTPσ but might also be inhibited by excessive transfection efficiencies. In addition, the AP probe method did not lend itself to straightforward quantitation of PTPσ-AP probe binding. There was both background AP activity, despite heat inactivation and a significant level of background probe binding to the cell line. In addition, there was significant inter-experimental variation in PTPσ expression level.

The role of homophilic binding in other RPTPs was not established using probe binding methods. Classically, techniques such as differential adhesion have been used to demonstrate homophilic adhesion (Brady-Kalnay et al., 1993; Gebbink et al., 1993b; Brady-Kalnay and Tonks, 1994; Sap et al., 1994; Zondag et al., 1995; Wang and Bixby, 1999; Baker et al., 2000b; Yang et al., 2003; Cismasiu et al., 2004; Yang et al., 2005; Aricescu et al., 2006). Experience suggested that the establishment of two stably transfected cell lines expressing distinctly tagged PTPσ proteins was a difficult proposition (A. Stoker, unpublished results). However, recent work on PTPμ has used an alternative method to assess homophilic adhesion (Cismasiu et al., 2004). Analysis of homophilic binding by PTPσ using SDS-PAGE confirmed that PTPσ extracellular domains bind to HEK-293T cells. This method was more easily quantifiable than the immunohistochemical method outlined above. However, in this case there was no evidence for a decrease or increase in PTP-AP probe binding following transfection with PTPσ. However, it may be that SDS-PAGE is an inappropriate method to use in this case. The method by definition takes an average of the probe binding across the whole plate. This would indeed be more sensitive to low levels of probe binding in the absence of any background binding. However, given that significant amounts of probe bind to untransfected cells, it would require a significantly greater level of probe binding following transfection to detect any change.

The experiments described in this thesis and those published previously do not therefore support a role for homophilic adhesion in PTPσ function (Haj et al., 1999). However, several interesting points can be made about these experiments. Firstly, it may be possible to obtain large amounts of the PTPσ-interacting protein present on HEK-293T cells and thus to potentially identify a novel PTPσ ligand. Although it is not clear whether HEK-293T cells express PTPσ (Stoker, 1994) and so whether this target could reflect a physiological PTPσ ligand, it would represent the first PTPσ ligand to be identified from a direct interaction in living cells. In addition, experiments in two cell lines seem to show that

the level of PTPσ-AP probe binding is affected by PTPσ transfection. Whilst the limitations of the AP-probe method are well known, this might suggest that either shed or cell membrane PTPσ interferes with PTPσ-AP probe binding. It is likely to become necessary to conduct differential adhesion experiments as used on those members of the RPTP family known to show homophilic adhesion. These experiments will probably require the use of PTPσ mutants that cannot undergo proteolysis and shedding from the cell membrane.

The shedding rate of PTPσ extracellular domains was serendipitously noted during the SDS-PAGE homophilic binding experiments. It appeared that the ectodomain shedding rate was significantly greater from cells expressing full-length PTPσ compared to the PTPσ-EC-AP or FNΔ3-AP constructs. Accumulation of satisfactory amounts of the AP fusion proteins required several days (Aricescu, 2002). After short incubations in conditioned media, a striking amount of shed PTPσ extracellular domain was detected (Figure 7.3 B). Despite this though the overall level of soluble protein was comparable at 3-4 days (Data not shown). Therefore, it is possible that shed PTPσ does not accumulate in conditioned media at the same rate at which it is shed, but that a significant proportion is lost from the media via degradation, binding to cells or the extracellular matrix or by endocytosis into cells. Interestingly, endocytosis would explain the unexplained portion of extracellular domain PTPσ protein that is protected from limited trypsin digestion. In addition, this high rate of shedding may suggest that expression of a tagged full-length PTPσ may be a more efficient method to produce soluble probe than expression of a truncated secreted protein such as the AP probe. Finally, although a significant proportion of PTPσ is detectable as the full-length form following expression in HEK-293T cells, in tissues PTPσ and other type IIa RPTPs are predominantly found in the form of the extracellular cleavage fragments (Stoker et al., 1995a; Stoker et al., 1995b). If PTPσ secretion from the cell does occur as rapidly as suggested by this data and previous pulse chase experiments on LAR, then much of the PTPσ detected in vivo may not be in the form of the E_{81}/P_{85} complex previously reported. Instead, large amounts of cleaved E_{81} protein may represent shed protein that, following shedding, has re-bound to an unknown receptor on the cell membrane or in the extracellular compartment (Yu et al., 1992; Streuli et al., 1992).

SDS-PAGE analysis of the His-tagged PTPσ extracellular domain deletion series revealed a number of unexpected high molecular weight bands (Figure 7.4 B). A number of possible explanations exist for these bands. Firstly, they may represent aggregates or

complexes of the His-tagged proteins. Such aggregates may be artefactual due to their high expression level (Di Fiore et al., 1987; Di Marco et al., 1990; Maru et al., 1990; Brandt-Rauf et al., 1990). However, similar species are not clearly seen with the endogenous full-length soluble extracellular domain protein although they would be similar in size to the full-length protein. Bands of approximately this size were seen occasionally in conditioned media samples from cells transfected with wild-type PTPσ (Data not shown). However, such samples were routinely discarded, as it was believed that these bands reflected contamination of the conditioned media with cell-membrane associated full-length protein. Alternatively, these bands may represent complexes of PTPσ with another protein. In this case, it is likely that the low level of these bands reflects a high affinity interaction that is not completely dissociated under the conditions employed for lysis and SDS-PAGE analysis. However, the size of the high molecular weight bands does not increase in proportion to the increasing size of the corresponding His-tagged PTPσ peptide. Therefore, if these proteins represent complexes of another protein with PTPσ, then one of two situations must exist. Either different proteins interact with each deletion mutant or the valency of the interaction must vary from one deletion mutant to the next. This raises a final possibility for the nature of the high molecular weight species. They may represent PTPσ homo-oligomers with different valencies. Since AP fusions are believed to form dimers, it is of note that the high molecular weight species seen with PTPσ-FNΔ3-AP.His is of the expected size for a PTPσ-FNΔ3-AP.His dimer. However, the His-tagged proteins that do not contain the AP moiety would not be constrained to dimer formation. The approximate sizes of the deletion series, the corresponding high molecular weight band and the suggested valency of the PTPσ homo-oligomer are as follows:

1. PTPσ-FNΔ2.His (55.8 kDa) - 150 kDa - ≈Trimer
2. PTPσ-FNΔ3.His (66.3 kDa) - 180kDa - ≈Trimer
3. PTPσ-FNΔ4.His (77.5 kDa) - 150kDa - ≈Dimer
4. PTPσ-FNΔ3-AP.His (120.6 kDa) - >200kDa - ≈Dimer

The nature of these homo-oligomeric complexes may be revealed as part of the ongoing biochemical characterisation of these proteins

Attempts were made to identify PTPσ substrates using wild type and substrate trapping forms of PTPσ. No interacting proteins could be identified using wild type PTPσ or substrate trapping PTPσ in the absence of phosphatase inhibitors but a number of interacting bands were visible following the immunoprecipitation of substrate trapping

PTPσ from tyrosine phosphatase inhibited lysates. Unfortunately, it did not prove possible to detect these coimmunoprecipitating bands above background on gels stained with silver or Brilliant Blue and so, it was not possible to identify these bands. During these experiments it was confirmed that PTPσ is preferentially tyrosine phosphorylated in the cleaved form rather than the FL form (Chapter 6). In addition, the level of tyrosine phosphorylation of PTPσ was increased by substrate trapping mutations, confirming that PTPσ, like other RPTPs, is able to auto-dephosphorylate itself (den Hertog et al., 1994; Tsujikawa et al., 2001; Aricescu, 2002). As this effect occurred in the presence of an irreversible phosphatase inhibitor in the lysis buffer, tyrosine dephosphorylation is likely to have occurred in the cell rather than to represent an artefactual post lysis effect. Finally, it is possible that the incorporation of non-native sequence (i.e. epitope tags) interferes with PTPσ protein-protein interactions. Recent work has identified an interaction of wild type PTPσ with certain growth factor receptors (Faux et al, manuscript in preparation).

Several factors were identified that might be responsible for the failure of the substrate trapping/immunprecipitation approach to produce sufficient amounts of PTPσ interacting proteins to permit their identification. Firstly, the level of PTPσ expression achieved was inadequate. Secondly, the specificity of the immunoprecipitation requires optimisation. In addition, although tyrosine phosphatase inhibition appeared to be required to generate sufficient phosphotyrosine substrate, it may block substrate binding by the trapping mutants. Attempts to minimise this effect by separating overexpressed PTPσ and the tyrosine phosphatase treatment in different cell cultures were not successful (Data not shown). Although some trapping mutations may be relatively immune to the conformational effects of catalytic cysteine oxidation, substitution at both catalytic site cysteines may be required for complete immunity. Alternative strategies might include overexpressing an active tyrosine kinase rather than using a phosphatase inhibitor to boost phosphotyrosine levels. A further problem with the substrate trapping mutations is that they are likely themselves to alter the conformation of the active site and so potentially interfere with substrate binding (Scapin et al., 2001). However, the substrate trapping mutations are an unfortunate necessity for substrate co-immunoprecipitation. In their absence, the substrate phosphotyrosine is rapidly hydrolysed and the substrate is ejected from the active site, preventing its co-immunoprecipitation (Guan and Dixon, 1991). A solution to this *Catch-22* situation is not immediately apparent and it is worth noting that although substrate trapping mutants have been extremely successful with non-receptor PTPs, they have been much less useful with RPTPs and there have been few successful

attempts to initially identify RPTP substrates using full length proteins expressed in cultured cells.

The substrate trapping approach was pursued using bacterially expressed GST-fusion proteins to avoid many of the problems outlined above. Several GST-PTPσ fusion proteins were designed for expression in *E.coli*; however, it proved difficult to obtain efficient expression of most of the GST-PTPσ fusion proteins. It was only possible to induce expression of two of the five GST-PTPσ fusion proteins (C215S, C215D) and experiments using these proteins are ongoing (C. Faux, unpublished data). Recent work optimising the expression of PTPσ proteins in E.coli may guide the production of other GST-PTPσ fusions (Lee et al., 2006b). It is of note that both of the proteins that were successfully expressed (C215S, C215D) lacked the D1 or membrane proximal catalytic cysteine (C215). Moreover, the C215D mutation, which is perceived to be less structurally disruptive than the C215S mutation, was more difficult to obtain, requiring lower temperatures for successful expression. Therefore, it is possible that an intact D1 catalytic cysteine is for some reason detrimental to efficient expression in this bacterial expression system. Unfortunately, the immunoprecipitation experiment suggested that the D181A mutant might be the most useful of the trapping mutants. If it proves necessary to use the catalytic cysteine retaining PTPσ substrate trapping mutations, it may prove necessary to change to an alternative expression system. However, the most likely explanation for the difficulties experienced in GST-PTPσ fusion protein expression is that the size of the 2-domain fusion (~98.4 kDa) is close to the upper limit of what is possible using this kind of bacterial expression system. Alternative approaches might include using smaller GST fusions containing only the membrane proximal phosphatase domains or alternative expression systems. However, this would potentially be at the expense of losing some physiological accuracy if, as has been suggested, the D2 domain participates in some way in the regulation of D1 (Streuli et al., 1990; Wang and Pallen, 1991; Johnson et al., 1992; Debant et al., 1996; Wu et al., 1997; Kashio et al., 1998; Felberg and Johnson, 1998; Buist et al., 2000; Felberg and Johnson, 2000; Tsujikawa et al., 2001; Krueger et al., 2003; Persson et al., 2004; Wang and Johnson, 2005).

In this chapter, attempts were made to isolate proteins that interact with PTPσ. Although it was not possible to identify any PTPσ interacting proteins, pilot experiments proved the concept that immunoaffinity purification methods could be used to obtain PTPσ interacting proteins. Future work will optimise the use of the tools generated in this chapter to identify these proteins. In addition, the role of homophilic interactions in PTPσ

function was revisited. Although it was not possible to demonstrate homophilic adhesion with PTPσ extracellular domains, data suggested that this area might fruitfully be probed in greater detail. Lastly, the PTPσ extracellular domain binds to a ligand on HEK-293T cells. Given the difficulty in identifying PTPσ interacting proteins, these cells represent an abundant source of a potential PTPσ ligand that should be followed up. If proven, this interaction would represent the first example of a PTPσ interacting protein demonstrated in living cells. In conclusion, the identification of PTPσ interacting proteins remains a key goal in developing our understanding of the function of this poorly understood protein and indeed of the large family of RPTPs, about which still little is understood.

Chapter 8: Cloning of chicken LAR(RPTPf)

In vertebrates, the type IIa family of RPTPs has three members: PTPδ, LAR (PTPf) and PTPσ. The phenotype produced by deletion of any one of these genes in mice is relatively mild with only PTPσ knockouts exhibiting significant neonatal mortality (Skarnes et al., 1995; Schaapveld et al., 1997; Elchebly et al., 1999; Wallace et al., 1999; Uetani et al., 2000). However, disruption of PTPσ and PTPδ together has a synergistic effect (Stepanek et al., 2005; Uetani et al., 2006). *Drosophila melanogaster* has six RPTPs of which four are selectively expressed in neurons (DLAR, DPTP69D, DPTP99A, DPTP10D), and a fifth (DPTP52F) is expressed only in neurons after formation of the ventral nerve cord (Streuli et al., 1989; Tian et al., 1991; Yang et al., 1991; Hariharan et al., 1991; Oon et al., 1993; Desai et al., 1994; Sun et al., 2000a; Schindelholz et al., 2001). Of these "neural" RPTPs, DLAR and arguably DPTP69D are structurally similar to type II RPTPs and as such are thought to share a common lineage with the vertebrate type IIa and IIb RPTPs (Schindelholz et al., 2001). Disruption of any one of the *Drosophila* neural RPTPs tends to produce only a mild, partially penetrant defect in neuronal development whereas the combined disruption of two or more fly RPTPs produces more striking defects (Desai et al., 1996; Krueger et al., 1996; Desai et al., 1997a; Garrity et al., 1999; Newsome et al., 2000a; Sun et al., 2000a; Sun et al., 2001; Schindelholz et al., 2001; Maurel-Zaffran et al., 2001; Clandinin et al., 2001; Kaufmann et al., 2002; Desai and Purdy, 2003). On the other hand, the functional relationship between fly RPTPs varies between developmental pathways such that in some systems, certain *Drosophila* RPTP mutations partially rescue the phenotype of mutations in other RPTPs. Therefore, it has been postulated that the fly RPTPs cooperate and oppose each other at multiple decision points in axon outgrowth (Desai et al., 1997a; Sun et al., 2001). The expression patterns of the vertebrate type II RPTPs overlap (1.2.1.2). Therefore, it is possible that as in *Drosophila*, vertebrate RPTPs act together to regulate axon guidance at choice points. This would potentially allow intact RPTPs to partially compensate for the disruption of a single RPTP gene such as PTPσ. Only recently have the first combined disruption studies been published using vertebrate models (Stepanek et al., 2005; Uetani et al., 2006). Together with the previously identified PTPσ and PTPδ, the isolation of the last remaining uncloned chick type IIa RPTP, LAR, will allow the potentially overlapping roles of type II RPTPs in axon guidance to be analysed.

Several different methods were used to isolate chick LAR. Degenerate PCR and library screening with PTPσ fragments were unsuccessful and only PTPσ sequences were obtained in this way. The isolation of the chick homologue of LAR was made possible by the concurrent development of several publicly available chicken EST databases. These contain a number of small LAR fragments, which allowed the design of LAR specific primers and subsequent successful RT-PCR. Two clones were obtained from independent experiments and sequenced. The clones encoded LAR isoform 1 with three immunoglobulin domains, seven (see below) fibronectin type III domains and two intracellular tyrosine phosphatase domains. The chick LAR clones were almost full-length and lacked only small regions at the 5' and 3' end of the coding sequence. These gaps were filled by *in silico* cloning from the EST databases to obtain a predicted sequence for the entire chick LAR protein.

8.1: Cloning

8.1.1: Library screen

Using a previously constructed library containing cDNA from day 7 chick embryos (Aricescu, 2002), plates were prepared and probed with a chick LAR EST probe. The probe was obtained by a Basic Local Alignment Search Tool (BLAST) search of a chick bursal EST database (DKFZ clone: dkfz426_27i10r1) (Altschul et al., 1990; Abdrakhmanov et al., 2000). The EST was a close match to the 3' untranslated region (UTR) of human LAR. The 3' EST was not conserved between type IIa RPTP subfamilies (i.e. PTPσ vs PTPδ vs LAR) but was highly conserved between species orthologues (i.e. mouse LAR vs human LAR; Data not shown). Thus, the probe was highly specific for LAR orthologues. However, library screening using replica plates was unsuccessful. Only chick PTPσ was isolated in these experiments. (Figure 8.1).

8.1.2: Degenerate PCR

Following the failure of the colony screening, the same chick library was probed using RTPCR with degenerate oligonucleotides. Primers were designed which had the best possible combination of low degeneracy with specificity for LAR orthologues over other type IIa RPTPs. Following a process of optimisation, a number of PCR products were generated. However, following cloning and sequence analysis, these all proved to be chick PTPσ (Figure 8.2).

Figure 8.1: cDNA library screening using colony hybridisation with a LAR 3' EST probe

Filter replicas were taken from agar plates plated with E.coli transformed with the chick cDNA library (R. Aricescu, unpublished data). The nylon filters were probed with a chick LAR 3'EST probe and a chemiluminescent detection system. Three representative filters are shown with positive colonies highlighted with a red circle. These colonies were identified on the original plates and subjected to DNA sequence analysis. All positive colonies analysed contained chick PTPσ. The black orientation markings at the edges of the plates and the filter grid system can also be seen.

8.1.3: Long-range PCR

During the course of this thesis, several chick genome and chick EST projects were established that gradually provided chick sequence data (Boardman et al., 2002; Carre et al., 2006). Several ESTs were identified using the BLAST tool that corresponded to chick LAR (Altschul et al., 1990). Using these sequences to design PCR primers, PCR was attempted on the library described above and on fresh chick cDNA. It was not possible to generate LAR product using the chick library. However, optimisation of the PCR conditions using chick cDNA (Wayne barnes, Personal communication) to optimise for the recovery of long, accurately replicated PCR products generated a single LAR specific band (Figure 8.3). This was cloned and sequenced using conventional methods. To allow accurate determination of the chicken LAR DNA sequence and to reveal errors introduced during the reverse transcription or PCR steps, a second LAR clone was generated in a completely independent experiment and the two sequences were compared.

8.2: Sequence analysis

8.2.1: DNA sequence

Using RT-PCR as described above, two independent clones of chicken LAR were isolated. The cDNA sequences are included in the appendix to this work (Appendix 1: cLAR DNA sequences). Slightly different primer sets were used for the two cloning experiments with the result that the two sequences differ slightly in length. The longer, clone 1, is 5381 base pairs long whereas the shorter, clone 2, contains a shorter region of 5243 base pairs entirely located within the boundaries of clone 1. The difference in size is largely accounted for by a single area of 5' sequence, present in clone 1 that is lacking in clone 2. Although the 5' primers used to amplify clones 1 and 2 were to all intents identical, clone 2 begins 131 base pairs after this shared primer site and lacks any primer-derived sequence at its 5' end. The reason for this difference is unclear.

A.

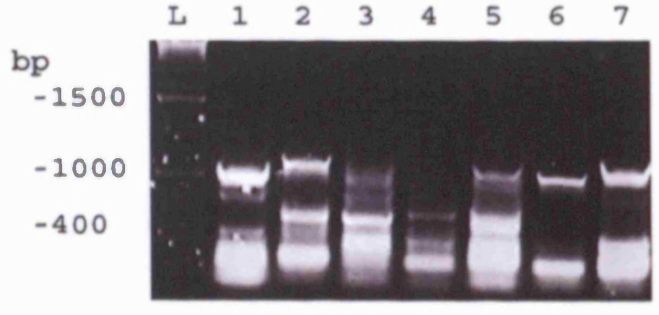
Codehop LAR 1

xLAR 550	c	gtg	gcc	tct	ttt	gtg	tgc	caa	gca	ac
mLAR 150	g	gtg	gcc	tcc	ttc	gtg	tgc	caa	gct	ac
1F	c	gtg	gcc	tcc	ttc	gtg	tgy	car	gcn	ac
		V		A		S		F		V		C		Q		A

Codehop LAR 1R (Reverse complement)

XLAR 1130	gaa	gtg	atg	cct	ggg	ggc	a
mLAR 730	gag	gtg	atg	ccg	ggc	ggc	a
1R	gar	gtn	atg	ccc	ggc	ggc	a
		E		V		M		P		G		G	

B.



C.

Figure 8.2: cDNA library screening using degenerate primers

A chicken cDNA library (Aricescu, 2002) was amplified using degenerate primers designed using the Consensus Degenerate Hybrid Oligonucleotide Primer (Codehop) system (http://bioinformatics.weizmann.ac.il/blocks/codehop.html) (Rose et al., 1998; Rose et al., 2003).

A. Illustrative alignments are shown of Codehop LAR 1 forward and reverse primers with *Xenopus laevis* (Accession number AF197945) and *Mus musculus* (AF300943) LAR to demonstrate the principle of the Codehop system.

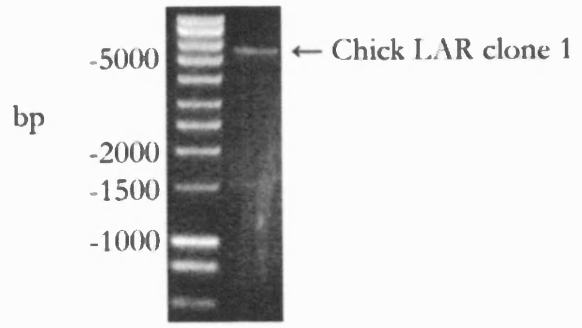
B.& C. Agarose gels showing the pattern of bands amplified by each set of Codehop primers using denaturation temperature of 50°C (B) or 45°C (C). Lanes 1, 2, etc = Codehop primer set 1F=1R, 2F+2R etc. L= Hyperladder 1 (Bioline) – molecular weights as indicated.

The two clones were sequenced bidirectionally and only two sequence variations were found between the two clones. Sequence variation one results in the replacement of a serine (Codon: tcg) residue with a proline (Codon: ccg) residue. The relevant nucleotide difference is LAR clone 1 thymine 2054 → LAR clone 2 cytosine 1923 (See appendix 1). Xenopus LAR has a serine (Codons: tca) at this site and human, mouse and rat homologues all have an alanine (Codons: gcn human gcc, mouse gct, rat gct) at this site. Sequence variation two results in a silent change in a proline codon (Codons: cca-ccg). Unfortunately, unlike sequence variation one, this residue is not conserved in all species and so it more difficult to predict which sequence is correct. For example, Xenopus LAR has an alanine at this site (Codon: gca);*Takifugu rubripes (Fugu)* LAR has a cysteine at this site (Codon tgt); rat (Codon: cct), mouse (Codon: cca) and dog (Codon: ccg) have a proline at this site and human LAR has a leucine (Codon: ttg). The relevant nucleotide difference is LAR clone 1 adenine 2452 → LAR clone 2 guanine 2321 (See appendix 1). In addition to comparison of the chicken LAR clone sequences at the sequence variation sites with LAR orthologues from other species, publicly available chick EST databases were examined for LAR EST clones that might be informative. ESTs were identified that covered the regions of interest; the EST sequences agreed with the LAR clone 1 sequence at both sites (Variation 1 (Codon tcg) – Genbank accession number XM_422408; Varation 2 (Codon cca) – ChEST101j13). Lastly, a short region at either end of each clone was derived from the PCR primers and as such remains to be verified empirically. However, several chick ESTs isolated independently from those used to design the oligonucleotide primers confirm that this sequence is correct.

8.2.2: Protein sequence

Domain analysis of the translation of the chicken LAR cDNA revealed that the clones encode the full-length isoform of chick LAR containing three Ig domains, seven (see below) fibronectin type 3 domains and two intracellular tyrosine phosphatase domains (Figure 8.4A). However, neither clone contains the full-length chicken LAR cDNA. By comparison to full-length LAR orthologues found in other species, clone 1 is missing approximately 70-80 amino acids (~220 base pairs) at its amino terminus. The carboxy-terminal end is missing about 20-30 amino acids (~75 base pairs).

A.



B.

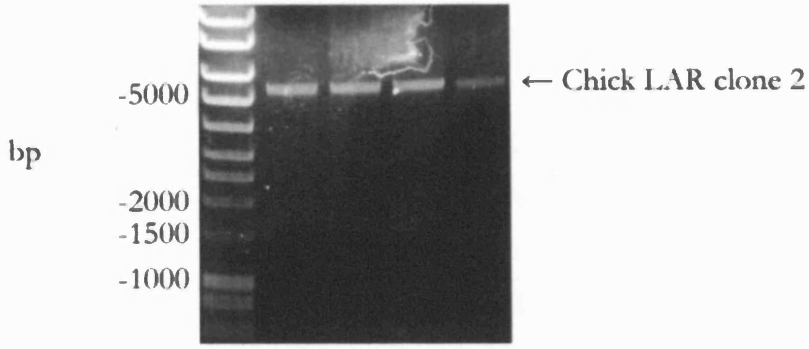


Figure 8.3: Long and accurate PCR (LA-PCR) cloning of chicken LAR

Using independently generated reagents, chicken LAR was cloned using long and accurate PCR on cDNA reverse transcribed from RNA derived from chick embryos.

A. RT-PCR cloning of chicken LAR clone 1

B. RT-PCR cloning of chicken LAR clone 2

8.2.3: Sequence extrapolation using ESTs from public databases

The LAR clone sequences did not contain the full-length chicken LAR cDNA. During the course of this thesis, significant amounts of chicken sequence data has been made publicly available as part of the work of two chicken EST sequencing consortia (Biotechnology and Biological Sciences Research Council/University of Manchester database – http://chick.umist.ac.uk/ (Boardman et al., 2002; Hubbard et al., 2005); University of Delaware – http://www.chickest.udel.edu/). Therefore, the available data was examined to determine whether it was possible to obtain the rest of the chicken LAR cDNA in this way.

Several cDNAs were identified that extended the chicken LAR clones at the 5' and 3' ends to complete the coding sequence. Firstly, at the 5' end, BLAST (Altschul et al., 1990) was used to probe the UMIST chick EST database with the LAR clone 1 sequence and two EST clones were retrieved (Umist database ref. nos. ChEST35h5, ChEST35p7 – Stage 20-21 whole embryo) that aligned with the extreme 5' end of clone 1. Translation of the consensus sequence of these 2 ESTs extended the LAR sequence to include the predicted ATG and Kozak consensus sequence. Similarly, at the 3' end a BLAST search of the UMIST database retrieved two independently derived EST clones (ChEST371m20 – Stage 36 limbs, ChEST958o6 – adult pancreas) that extended the 3' end of clone 1. Translation of the resulting consensus sequence extended the LAR sequence to include the termination codon.

8.2.4: Analysis

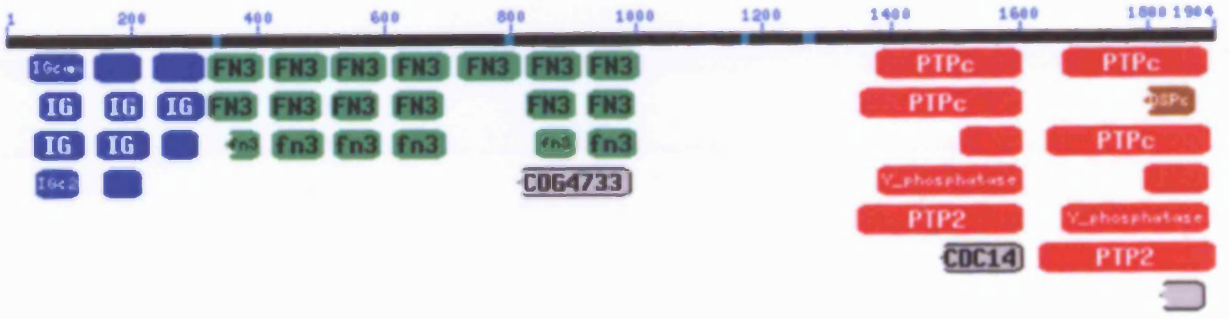
The completed chicken LAR protein sequence (Derived from LAR clone 1 + EST clones as described above) was analysed using standard bioinformatic techniques. The identity of chicken LAR as the chicken LAR homologue was confirmed in a number of ways. Firstly, domain prediction using CD-Search (Marchler-Bauer and Bryant, 2004; Marchler-Bauer et al., 2005) confirmed that chicken LAR has the characteristic architecture of a type IIa RPTP. That is, chicken LAR contains three amino-terminal extracellular Immunoglobulin (Ig) domains followed by a number of fibronectin type III (FNIII) domains and paired, carboxy-terminal intracellular tyrosine phosphatase domains (Figure 8.4A). Interestingly it was noted that CD-search only identified 7 FNIII repeats in chicken LAR. A similar number were identified in LAR orthologues from other species, whereas type 1 isoform PTPσ and PTPδ clones were shown to have the expected 8 FNIII domains.

It is also interesting to note that PTPσ and PTPδ show significant conservation in the region between FNIII domain 7 and the transmembrane domain, which in these proteins contains an additional FNIII domain. In contrast, this region in LAR proteins shows substantial differences from the PTPσ/δ consensus (Figure 8.5). Some domain analysis prediction algorithms predicted an eighth FNII domain in this region of LAR but with significant differences from the model FNIII domain (Data not shown).

Analysis of the completed chicken LAR cDNA revealed that with respect to previously reported type IIa RPTP alternatively spliced exons it is LASE-a/b/d negative, LASE-c/meC positive, meA/B negative (Zhang and Longo, 1995; Pulido et al., 1995a).

Secondly, phylogenetic comparison of the DNA sequence of the membrane proximal tyrosine phosphatase domain confirmed that chicken LAR was more closely related to LAR family members and particularly *Fugu* LAR than PTPσ/δ family members (Figure 8.4B). The membrane proximal or sole tyrosine phosphatase domain was identified by comparison to the conserved domain database (Marchler-Bauer et al., 2005) tyrosine phosphatase consensus domain (CD00047.3/28929).

A.


1
200
400
600
800
1000
1200
1400
1600
1800 1904
IG
FN3
fn3
CDC4733
PTPc
V_phosphatase
PTP2
CDC14

B.

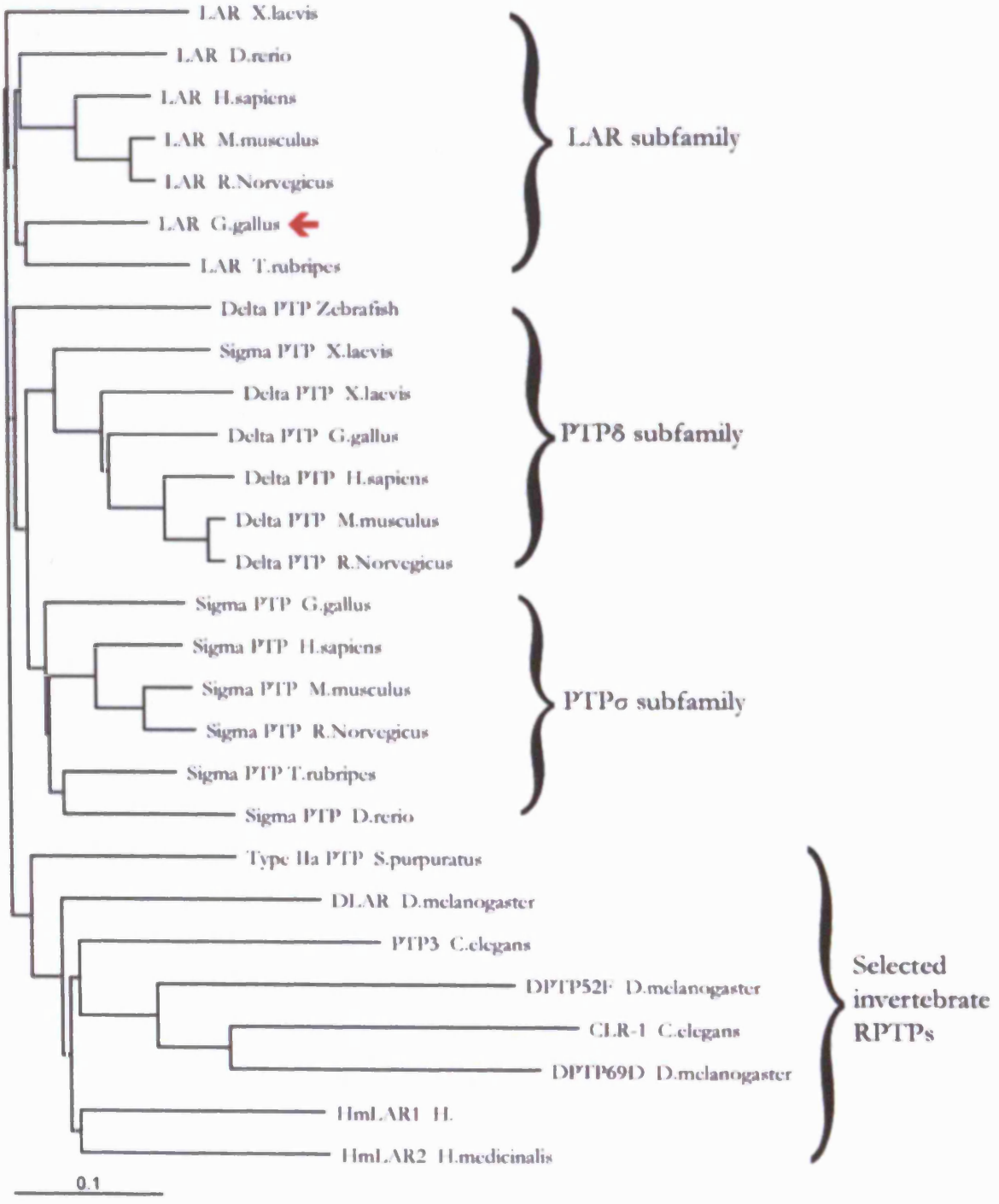

LAR X.laevis
LAR D.rerio
LAR H.sapiens
LAR M.musculus
LAR R.Norvegicus
LAR G.gallus
LAR T.rubripes
LAR subfamily
Delta PTP Zebrafish
Sigma PTP X.laevis
Delta PTP X.laevis
Delta PTP G.gallus
Delta PTP H.sapiens
Delta PTP M.musculus
Delta PTP R.Norvegicus
PTPδ subfamily
Sigma PTP G.gallus
Sigma PTP H.sapiens
Sigma PTP M.musculus
Sigma PTP R.Norvegicus
Sigma PTP T.rubripes
Sigma PTP D.rerio
PTPσ subfamily
Type IIa PTP S.purpuratus
DLAR D.melanogaster
PTP3 C.elegans
DPTP52F D.melanogaster
CLR-1 C.elegans
DPTP69D D.melanogaster
HmLAR1 H.
HmLAR2 H.medicinalis
Selected invertebrate RPTPs
0.1

Figure 8.4: Domain and phylogenetic analysis of chick LAR1

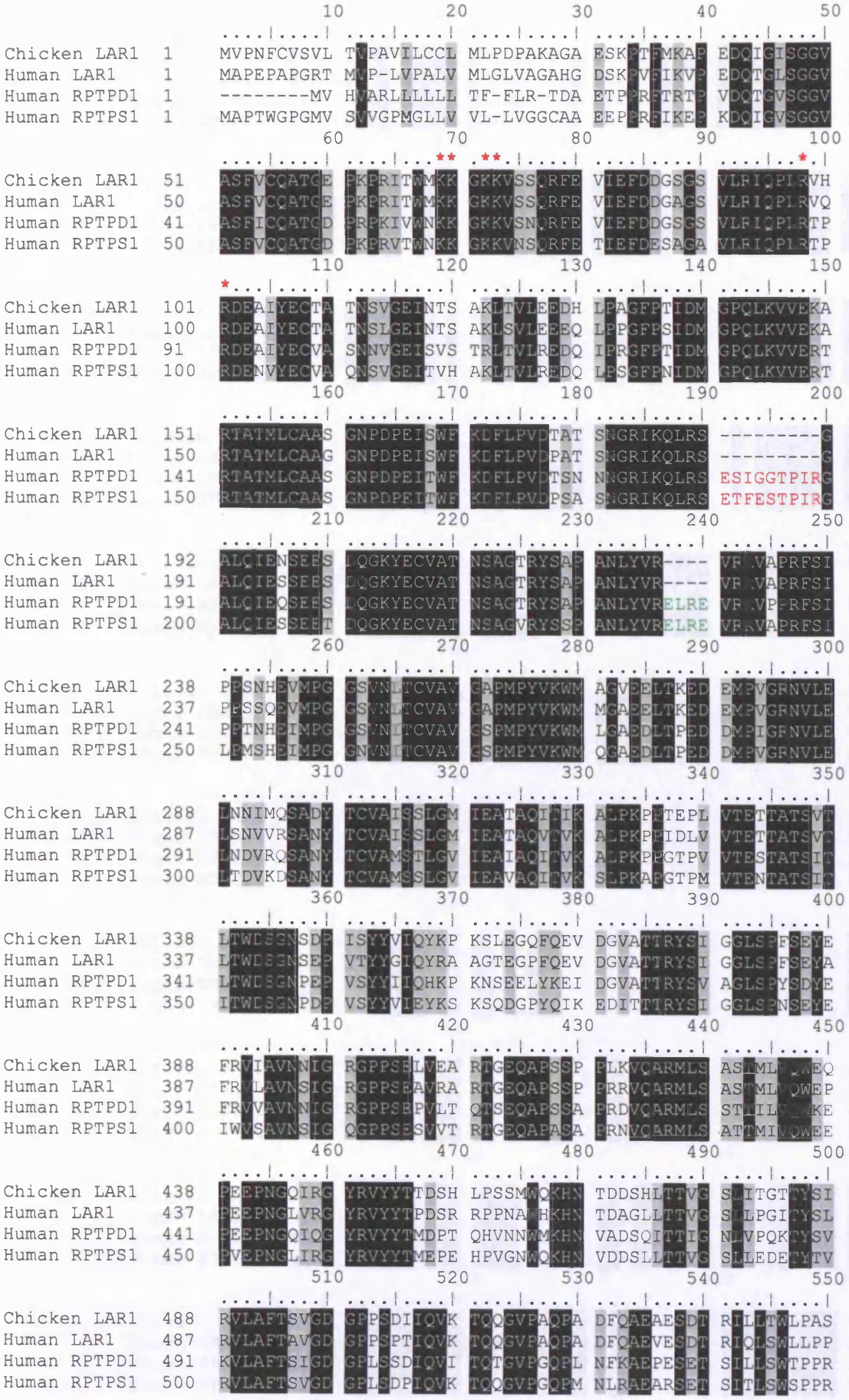
A. The domain structure of chick LAR1 was predicted using CD-Search, which predicts domains in submitted proteins by comparison to protein domain databases. Each row indicates the results obtained using a different protein domain database. Blue boxes indicate type 2 Ig domains, green boxes indicate FN3 domains and the red boxes indicate cysteine-based PTP domains. The grey box indicates regions of low similarity to specific known domains. The orange-brown box indicates a low level of homology to a DSP type PTP domain.
B. Phlyogenetic analysis of type II and related RPTPs based upon an alignment of membrane proximal or unique tyrosine phosphatase catalytic domains. Tyrosine phosphatase catalytic domains were defined according to the conserved domain database (CD00047.3). Alignment was performed using ClustalW (Thompson et al., 1994) and phylogenetic trees drawn using Phylip and Treedraw. Scale bar represents 0.1 substitutions per site. Chicken LAR is highlighted with a red arrow. PTP sequences used were as follows (Genbank accession numbers):

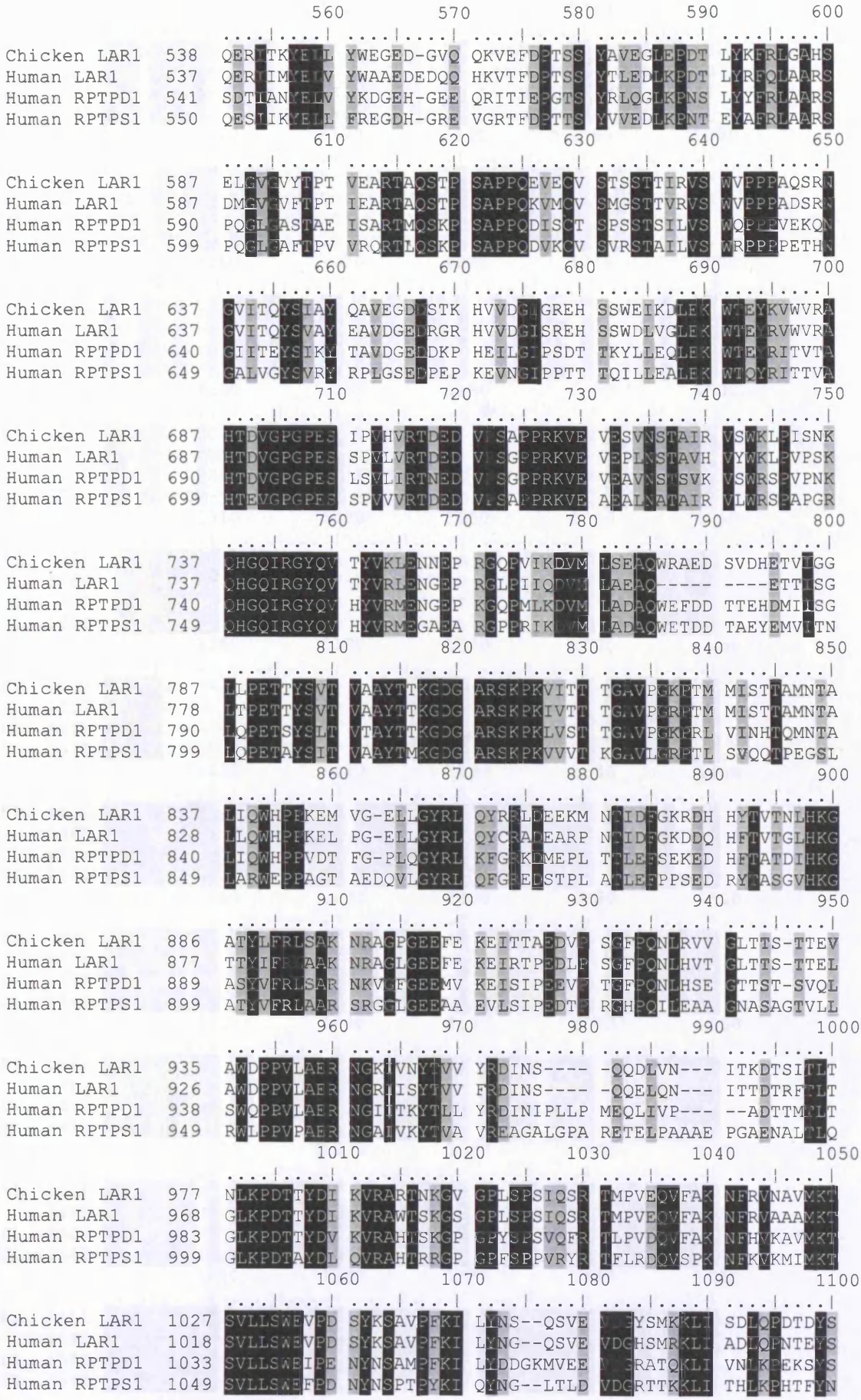
1. LAR (*Homo sapiens* – NM_002840, *Mus musculus* – NM_011213.1, *Rattus norvegicus* – NM_019249, *Danio rerio* – XM_684112, *Xenopus laevis* – AF197945, *Takifugu rubripes* – NEWSINFRUG00000161578)
2. PTPδ (*H.sapiens* – NM_130391, *M. musculus* – NM_001014288, *R. Norvegicus* – XM_233065, *D. rerio* XM_685632, *G. gallus* – XM_429156, *X.laevis* – AF197944
3. PTPσ (*H.sapiens* – NM_002850, *M. musculus* – NM_011218, *R.norvegicus* – L11587, *G. gallus* – NM_205407, *X. laevis* – AF198450, *T. rubripes* – NEWSINFRUG00000143678, *D. rerio* – XM_695020
4. Selected invertebrate RPTPs

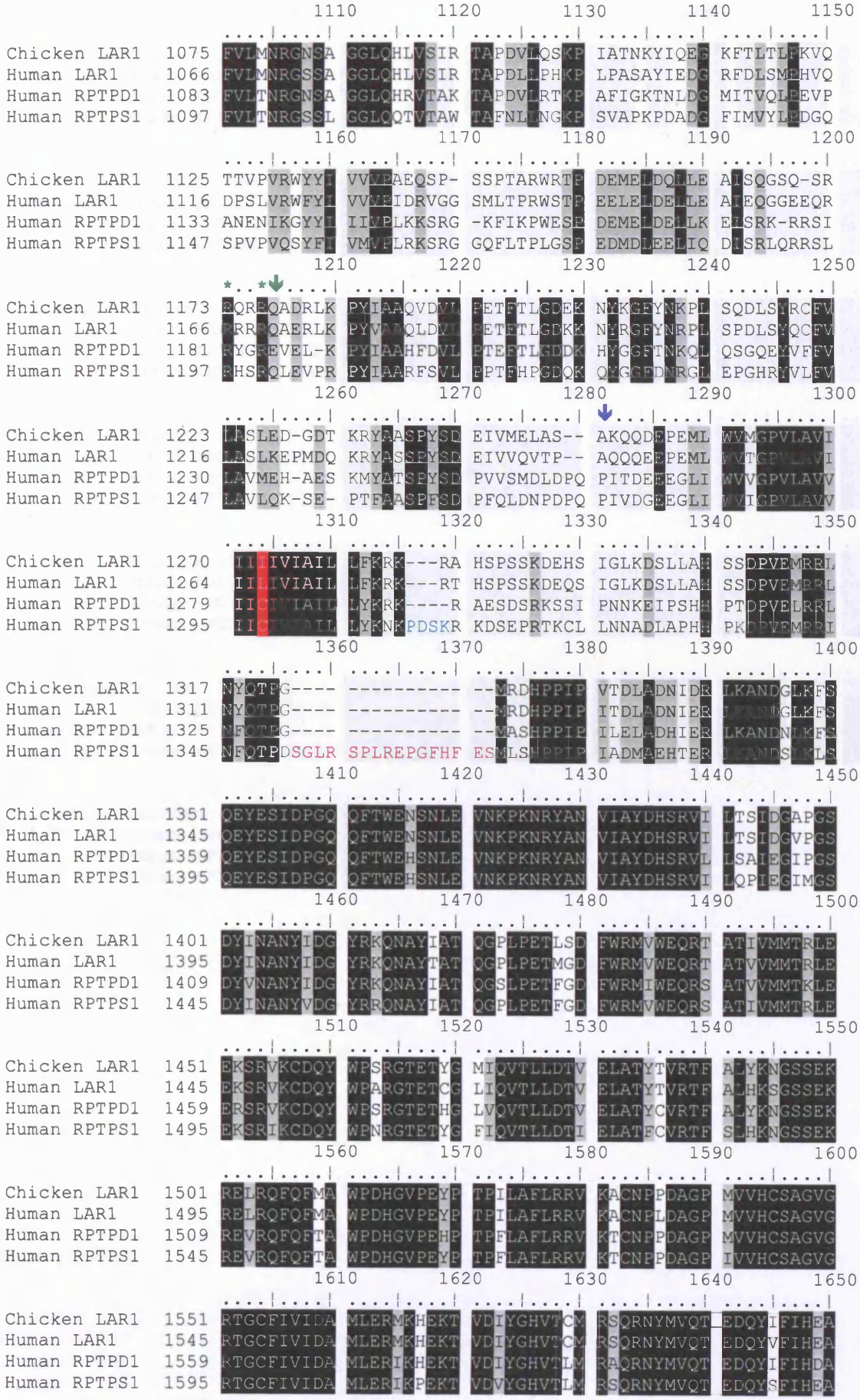
    a. *Strongylocentrotus purpuratus* unique type II RPTP – XM_780807)
    b. *C. elegans* PTP3 – AF316539, CLR-1 – AF047880
    c. *Drosophila* LAR – AH006566, PTP52F – NM_137249, DPTP69D – NM_176324
    d. *Hirudo medicinalis* HmLAR1 – AF017084, HmLAR2 – AF017083

Lastly, the complete chicken LAR protein sequence was aligned with those of human PTPσ, PTPδ and LAR proteins (Figure 8.5). This confirmed that chicken LAR was indeed the chick LAR orthologue rather than a PTPσ or PTPδ family member. Previous work on different members of the type IIa family has identified several important sequence motifs. The identity of these residues in chicken LAR was analysed using an alignment (ClustalW) of the completed LAR sequence with the human type II RPTP paralogues. Firstly, a number of basic residues located in the first and second Ig domains of chick PTPσ are important for binding to the only known type IIa ligand, heparan sulphate proteoglycans (Aricescu et al., 2002). These residues are absolutely conserved in chick LAR (Figure 8.5, red asterisks). Secondly, two arginine residues within a penta-arginine motif are essential for amino-terminal cleavage of human LAR (Serra-Pages et al., 1994). These residues are also present in chicken LAR (Figure 8.5, green asterisks, green arrow indicates cleavage site). However, the site at which human PTPσ undergoes amino-terminal cleavage (Aicher et al., 1997) is not conserved in human or chicken LAR (Figure 8.5, blue arrow indicates cleavage site). Lastly, as mentioned above, the transmembrane cysteine seen in all PTPσ or PTPδ proteins, but which is absent in all LAR proteins is indeed lacking in chicken LAR (Figure 8.5, red text).

Although this gene has been named chicken LAR in accordance with current practice, it is acknowledged that it might now be more appropriate to rename these genes according to the system used for all other RPTPs. This was originally proposed in the paper that set forward the type 1-5 (now 8) classification for RPTPs, but was not acted upon (Fischer et al., 1991). In addition to simplifying the classification of PTPs, it is probably no longer appropriate to describe this gene by virtue of its similarity to the leucocyte common antigen or CD45. This analogy was initially made on the basis of the shared tandem phosphatase domains (Streuli et al., 1988). However, it is now apparent that as judged by this criterion, most RPTPs are at least as equally closely related to CD45 as is LAR. Thus, it may be argued that LAR genes should be renamed PTPf or RPTPf in accordance with the nomenclature used in the genome projects.


Chicken LAR1
Human LAR1
Human RPTPD1
Human RPTPS1


Chicken LAR1
Human LAR1
Human RPTPD1
Human RPTPS1


1110 1120 1130 1140 1150
Chicken LAR1 1075 FVLMNRGNSA GGLQHLVSIR TAPDVLQSKP IATNKYIQEG KFTLTLFKVQ
Human LAR1 1066 FVLMNRGSSA GGLQHLVSIR TAPDLLPHKP LPASAYIEDG RFDLSMPHVQ
Human RPTPD1 1083 FVLTNRGNSA GGLQHRVTAK TAPDVLRTKP AFIGKTNLDG MITVQLPEVP
Human RPTPS1 1097 FVLTNRGSSL GGLQQTVTAW TAFNLLNGKP SVAPKPDADG FIMVYLPDGQ
1160 1170 1180 1190 1200
Chicken LAR1 1125 TTVPVRWYYI VVIPAEQSP- SSPTARWRTP DEMELDQLLE AISQGSQ-SR
Human LAR1 1116 DPSLVRWFYI VVIPIDRVGG SMLTPRWSTP EELELDELLE AIEQGGEEQR
Human RPTPD1 1133 ANENIKGYYI IINPLKKSRG -KFIKPWESP DEMELDELLK EISRK-RRSI
Human RPTPS1 1147 SPVPVQSYFI VMVPLRKSRG GQFLTPLGSP EDMDLEELIQ DISRLQRRSL
1210 1220 1230 1240 1250
Chicken LAR1 1173 RQRRQADRLK PYIAAQVDVL PETFTLGDEK NYKGFYNKPL SQDLSYRCFV
Human LAR1 1166 RRRRQAERLK PYVATQLDVL PETFTLGDKK NYRGFYNRPL SPDLSYQCFV
Human RPTPD1 1181 KYGKEVEL-K PYIAAHFDVL PTEFTLGDDK HYGGFTNKQL QSGQEYVFFV
Human RPTPS1 1197 RHSRQLEVPR PYIAARFSVL PPTFHPGDQK QYGGFDNRGL EPGHRYVLFV
1260 1270 1280 1290 1300
Chicken LAR1 1223 LASLED-GDT KRYRASPYSD EIVMELAS-- AKQQDFPEML WVMGPVLAVI
Human LAR1 1216 LASLKEPMDQ KRYASSPYSD EIVVQVTP-- AQQQEFPEML WVTGPVLAVI
Human RPTPD1 1230 LAVMEH-AES KMYATSPYSD PVVSMDLDPQ PITDEFEGLI WVVGPVLAVV
Human RPTPS1 1247 LAVLQK-SE- PTFAASPFSD PFQLDNPDPQ PIVDGFEGLI WVIGPVLAVV
1310 1320 1330 1340 1350
Chicken LAR1 1270 IIIIVIAIL LFKRK---RA HSPSSKDEHS IGLKDSLLAH SSDPVEMRRL
Human LAR1 1264 IILIVIAIL LFKRK---RT HSPSSKDEQS IGLKDSLLAH SSDPVEMRRL
Human RPTPD1 1279 IICIVIAIL LYKRK----R AESDSRKSSI PNNKEIPSHH PTDPVELRRL
Human RPTPS1 1295 IICIVIAIL LYKNKPDSKR KDSEPRTKCL LNNADLAPHH PKDPVEMRRI
1360 1370 1380 1390 1400
Chicken LAR1 1317 NYQTPG---- ---------- --MRDHPPIP VTDLADNIDR LKANDGLKFS
Human LAR1 1311 NYQTPG---- ---------- --MRDHPPIP ITDLADNIER LKANDGLKFS
Human RPTPD1 1325 NFQTPG---- ---------- --MASHPPIP ILELADHIER LKANDNLKFS
Human RPTPS1 1345 NFQTPDSGLR SPLREPGFHF ESMLSHPPIP IADMAEHTER LKANDSIKLS
1410 1420 1430 1440 1450
Chicken LAR1 1351 QEYESIDPGQ QFTWENSNLE VNKPKNRYAN VIAYDHSRVI LTSIDGAPGS
Human LAR1 1345 QEYESIDPGQ QFTWENSNLE VNKPKNRYAN VIAYDHSRVI LTSIDGVPGS
Human RPTPD1 1359 QEYESIDPGQ QFTWEHSNLE VNKPKNRYAN VIAYDHSRVL LSAIEGIPGS
Human RPTPS1 1395 QEYESIDPGQ QFTWEHSNLE VNKPKNRYAN VIAYDHSRVI LQPIEGIMGS
1460 1470 1480 1490 1500
Chicken LAR1 1401 DYINANYIDG YRKQNAYIAT QGPLPETLSD FWRMVWEQRT ATIVMMTRLE
Human LAR1 1395 DYINANYIDG YRKQNAYTAT QGPLPETMGD FWRMVWEQRT ATVVMMTRLE
Human RPTPD1 1409 DYVNANYIDG YRKQNAYIAT QGSLPETFGD FWRMIWEQRS ATVVMMTKLE
Human RPTPS1 1445 DYINANYVDG YRRQNAYIAT QGPLPETFGD FWRMVWEQRS ATIVMMTRLE
1510 1520 1530 1540 1550
Chicken LAR1 1451 EKSRVKCDQY WPSRGTETYG MIQVTLLDTV ELATYTVRTF ALYKNGSSEK
Human LAR1 1445 EKSRVKCDQY WPARGTETCG LIQVTLLDTV ELATYTVRTF ALHKSGSSEK
Human RPTPD1 1459 ERSRVKCDQY WPSRGTETHG LVQVTLLDTV ELATYCVRTF ALYKNGSSEK
Human RPTPS1 1495 EKSRIKCDQY WPNRGTETYG FIQVTLLDTI ELATFCVRTF SLHKNGSSEK
1560 1570 1580 1590 1600
Chicken LAR1 1501 RELRQFQFMA WPDHGVPEYP TPILAFLRRV KACNPPDAGP MVVHCSAGVG
Human LAR1 1495 RELRQFQFMA WPDHGVPEYP TPILAFLRRV KACNPLDAGP MVVHCSAGVG
Human RPTPD1 1509 REVRQFQFTA WPDHGVPEHP TPFLAFLRRV KTCNPPDAGP MVVHCSAGVG
Human RPTPS1 1545 REVRQFQFTA WPDHGVPEYP TPFLAFLRRV KTCNPPDAGP IVVHCSAGVG
1610 1620 1630 1640 1650
Chicken LAR1 1551 RTGCFIVIDA MLERMKHEKT VDIYGHVTCM RSQRNYMVQT EDQYIFIHEA
Human LAR1 1545 RTGCFIVIDA MLERMKHEKT VDIYGHVTCM RSQRNYMVQT EDQYVFIHEA
Human RPTPD1 1559 RTGCFIVIDA MLERIKHEKT VDIYGHVTLM RAQRNYMVQT EDQYIFIHDA
Human RPTPS1 1595 RTGCFIVIDA MLERIKPEKT VDVYGHVTLM RSQRNYMVQT EDQYSFIHEA

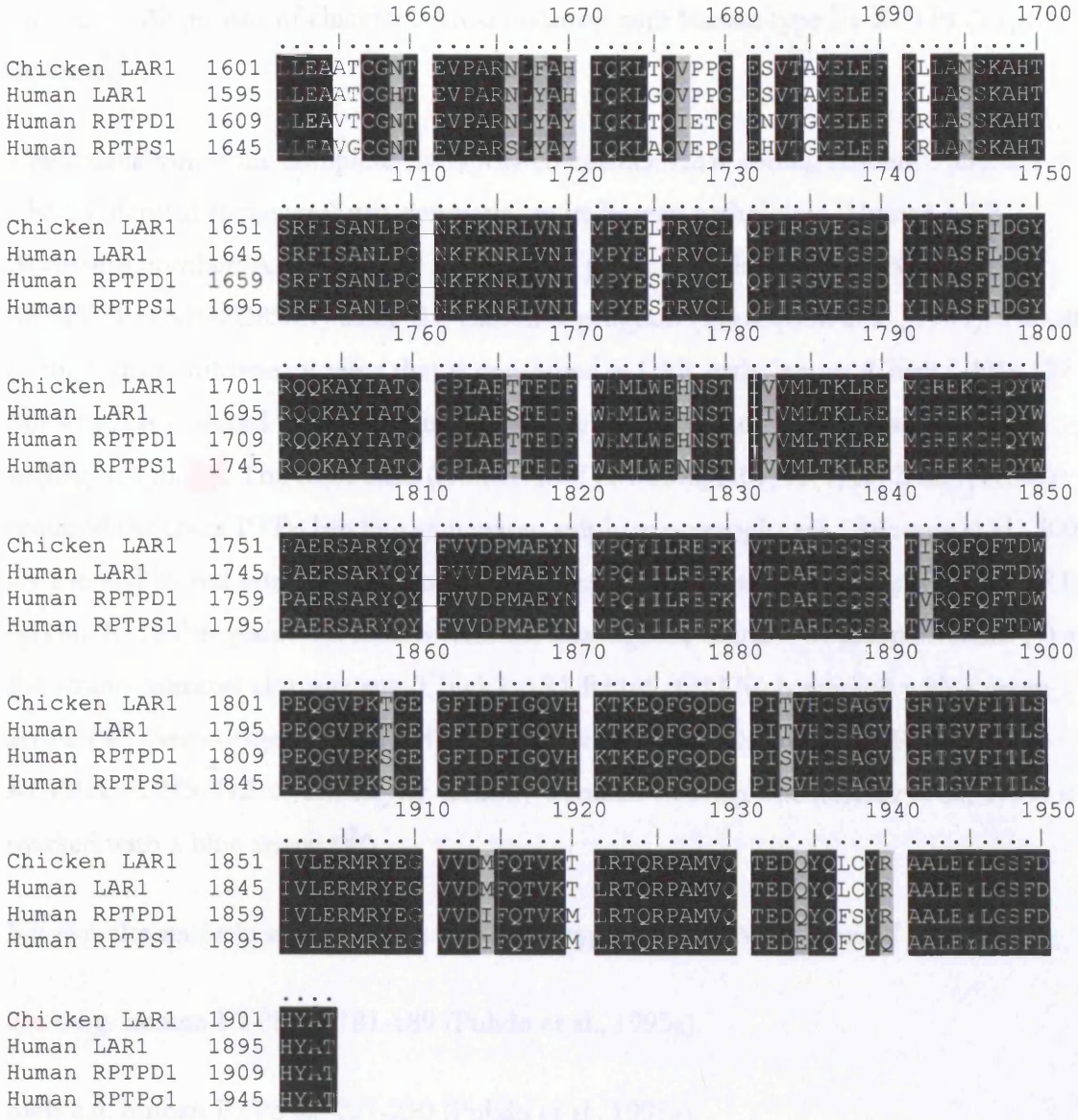

1660 1670 1680 1690 1700
Chicken LAR1 1601
Human LAR1 1595
Human RPTPD1 1609
Human RPTPS1 1645
1710 1720 1730 1740 1750
Chicken LAR1 1651
Human LAR1 1645
Human RPTPD1 1659
Human RPTPS1 1695
1760 1770 1780 1790 1800
Chicken LAR1 1701
Human LAR1 1695
Human RPTPD1 1709
Human RPTPS1 1745
1810 1820 1830 1840 1850
Chicken LAR1 1751
Human LAR1 1745
Human RPTPD1 1759
Human RPTPS1 1795
1860 1870 1880 1890 1900
Chicken LAR1 1801
Human LAR1 1795
Human RPTPD1 1809
Human RPTPS1 1845
1910 1920 1930 1940 1950
Chicken LAR1 1851
Human LAR1 1845
Human RPTPD1 1859
Human RPTPS1 1895
Chicken LAR1 1901
Human LAR1 1895
Human RPTPD1 1909
Human RPTPS1 1945

Figure 8.5 Alignment of chicken LAR translation with human type IIa RPTPs (Type I isoforms)

The translation of the complete, extrapolated chicken LAR coding sequence (clone 1 + ChEST-derived sequences) was compared to its human orthologue (Human LAR1 – Accession number: AAH48768.1) and type IIa paralogues (hRPTPσ1 – NP 002841.2; hRPTPδ1 – NP 002830.1) using the ClustalW program (Thompson et al., 1994). The site of the transmembrane cysteine that is conserved in LAR orthologues (Chick LAR1 1273) but which is replaced with isoleucine or leucine in all PTPσ or PTPδ orthologues is highlighted in red. The basic sites (Human RPTPσ1 K68, K69, K71, K72; R97, R100) required for chick PTPσ binding to heparan sulphate proteoglycans (Aricescu et al., 2002) are marked by red asterisks (*) above the sequence. The arginine residues (Chick LAR1 R1166, R1169) required for amino-terminal cleavage are marked by green asterisks (*) and the amino-terminal cleavage site (Chick LAR1 R1169/Q1170) is marked with a green arrow (⬇) (Serra-Pages et al., 1994). Finally. the site at which human PTPσ (Human RPTPσ1 P1275/I1276) undergoes carboxy-terminal cleavage site (Aicher et al., 1997) is marked with a blue arrow (⬇).

Known alternatively spliced exons present in type IIa RPTPs are marked as follows:

meA e.g. human PTPδ aa: 181-189 (Pulido et al., 1995a).

meB e.g. human PTPδ aa: 227-230 (Pulido et al., 1995a).

LASE-a e.g. human PTPσ aa: 1351-1366 (Zhang and Longo, 1995).

LASE-b e.g. human PTPσ aa: 1310-1313 (Zhang and Longo, 1995).

8.3: Discussion

This chapter describes the cloning of the third member of the chick type IIa RPTP subfamily, chick LAR (RPTPf). Several unsuccessful attempts to clone the chick LAR cDNA were described, with the cDNA finally being obtained using long and accurate RT-PCR. Two independent clones were obtained to allow the accurate determination of the chick LAR sequence. Two sequence variations were identified between the two clones but comparison to EST databases suggested that these represented amplification errors in clone 2 rather than genuine polymorphisms. The LAR clones encode the full-length or type 1 LAR isoform containing three extracellular amino-terminal immunoglobulin domains and 7 fibronectin type III repeats followed by the intracellular tandem tyrosine phosphatase domains. Phylogenetic analysis of the chick LAR gene confirmed that it was a member of the LAR sub-family, being most closely related to the *Takifugu rubripes* LAR protein. The LAR clones isolated are not complete and EST clones that will allow their completion are listed in the text.

As mentioned above, the cloning of LAR was problematic. Several possible explanations present themselves. Initial experiments were carried out using an existing cDNA library. The probe used for the initial library screen did not prove on final analysis to come from the chicken LAR gene. However, it did not prove possible to isolate LAR from the library even using the primers that were finally successful in cloning LAR. This suggests either that LAR was not expressed at a high enough level in the tissue of origin of the library or that the cDNAs were not long enough to span the 5 kilobase gap between the primer binding sites. As the library has previously been used to clone PTPσ and other similarly large genes, it is likely that LAR was not present in the library (Personal communication, R. Aricescu). The high degree of similarity between PTPσ and LAR probably explains why on low stringency screens, PTPσ was cloned with LAR specific primers. Although, LAR expression data is not available in the chick, analysis of data in other species suggests that many tissues express a combination of the type IIa RPTPs. However, in many tissues the expression level of PTPσ in particular is significantly higher than that of LAR. It is notable that the original cloning strategy used to isolate CRYPα/chick PTPσ used a LAR probe. Thus, the difference in expression level between PTPσ and LAR in chick embryos has previously resulted in the preferential isolation of PTPσ rather than LAR cDNAs (Stoker, 1994).

As described above, previous work on different members of the type IIa family has identified several important sequence motifs (Figure 8.5). Firstly, the binding site that is responsible in chick PTPσ for heparan sulphate proteoglycan binding is conserved (Aricescu et al., 2002). This suggests that chick LAR is able to bind heparin sulphate and therefore is likely to utilise the same (or closely related) physiological ligands as PTPσ. The ability of chicken LAR to bind HSPGs is easily assessable using the Receptor Affinity Probe system previously used to identify chick PTPσ as an HSPG-binding protein. It will be interesting to determine whether chick LAR, PTPσ and PTPδ bind the same ligands or have distinct specificities for different HSPGs.

Secondly, the furin-like amino-terminal proteolytic site (Serra-Pages et al., 1994) in LAR proteins is conserved suggesting that like other type II RPTPs, chick LAR will be subject to this kind of cleavage event. However, the region containing the carboxy-terminal metalloprotease type site in human PTPσ (Aicher et al., 1997) whilst highly conserved in all PTPσ and PTPδ proteins is not conserved in human or chick LAR1. Human LAR protein undergoes shedding in an analogous manner to that of human PTPσ (Streuli et al., 1992). It is may therefore be suggested that LAR carboxy-terminal cleavage is mediated by different mechanisms than that of PTPσ or PTPδ. However, the metalloproteases responsible for cleavage at this site do not have canonical cleavage sites and indeed do not always cleave at a specific site. Their specificity seems to be directed at a set distance from the plasma membrane rather than an amino acid sequence. Therefore, LAR cleavage may utilise the same enzymes despite the significant sequence variations.

Lastly, the transmembrane cysteine seen in all PTPσ or PTPδ proteins, but which is absent in all LAR proteins is indeed lacking in chick LAR1. Although mutational analysis of this residue in chick PTPσ did not show a role for this residue in the formation of high molecular weight species by wild type PTPσ, its striking pattern of conservation merits further study when the mechanism of type IIa RPTP dimerisation is better understood.

The sequence of chicken LAR, together with those of the previously determined chick PTPδ and PTPσ proteins will be important in the development of the understanding of the combinatorial role of the type II RPTPs. It has so far been very difficult to determine the role PTPs play in axon guidance. In different projections, they may play opposing roles and disruption of individual family members often has very little effect. The chick retinotectal projection is a well-known model for axon guidance. The ability to

manipulate all 3 members of an RPTP subfamily in an accessible system may help clarify the type of role PTPs play in cell signaling.

Chapter 9: Final discussion

The work in this thesis may be divided into three main categories. It was shown for the first time that a type IIa RPTP forms dimers in cell membranes (Lee et al., 2006a). In addition, the processing and quaternary structure of PTPσ was investigated. These data raised a number of interesting possibilities and suggested a new model for type IIa RPTP function. Secondly, a number of pilot experiments were conducted to identify the biochemical pathways with which PTPσ interacts. Unfortunately, no PTPσ-interacting proteins were identified. However, in agreement with studies on the type IV RPTP, PTPα, PTPσ was shown to form high molecular weight species in response to H_2O_2 treatment. It may be possible to adapt this finding to produce a useful assay that can in turn be used to follow the effects of other manipulations on PTPσ. In addition, the final member of the chick type IIa RPTP family was cloned to permit future experiments where members of the type IIa RPTP family are perturbed individually or in combination.

A large proportion of PTPσ was shown to be dimeric using the disulphide crosslinking technique (4.3). Although this may reflect a dominant effect of the ectopic cysteine residues, the finding that wild type PTPσ co-immunoprecipitates in the absence of any cellular treatments suggests that dimerisation is an important part of PTPσ function (Chapter 5). However, it has proven difficult to discover what effect dimerisation has on PTPσ function or indeed, whether mature PTPσ is constitutively dimeric. Preliminary experiments suggested that heparin sulphate, a putative PTPσ ligand that binds to the PTPσ extracellular domain, does not affect the degree of PTPσ dimerisation (6.5). However, it has recently been shown that heparin sulphate ligands only bind to the dimeric form of PTPσ. Therefore, it is possible that some PTPσ ligands do not affect PTPσ dimerisation *per se* but rather have allosteric effects on the conformation of receptor dimers or perhaps facilitate the presentation of an as yet unidentified ligand to the PTPσ dimer.

This thesis also presented some interesting findings with regard to type IIa RPTP cleavage. Firstly, in agreement with other studies, amino-terminal cleavage was shown to be dispensible for subsequent carboxyl-terminal cleavage or ectodomain shedding. In addition, carboxyl-terminal cleavage alone was shown to be insufficient for ectodomain shedding. This suggests that either an additional cleavage event or a conformational change is required for ectodomain shedding. It is of note that γ-secretase cleavage has recently been shown to play a role in PTPκ processing, although not in PTPκ ectodomain shedding

(Anders et al., 2006). It may prove possible using PTPσ mutants to dissect the molecular determinants and mechanism of RPTP ectodomain shedding.

The sensitivity of PTPσ species to extracellular trypsin suggested that a significant proportion of the E_{81} species, which is produced by amino-terminal cleavage, is located inside the cell. In contrast, most of the E_{90} species and the full length PTPσ appeared to be located at the cell surface, whether in the monomeric or dimeric form (4.2.2, 3.2.2). The PTPσ dimers described above contained exclusively either full-length PTPσ or proteolytically cleaved forms and full-length/cleaved heterodimers were not seen. Therefore, either PTPσ cleavage must affect both dimer subunits essentially simultaneously or only monomeric PTPσ is susceptible to cleavage, with the cleaved subunits retaining the potential of the parent molecule to form dimers.

These data suggest a model where full length PTPσ (FL_{166}) is expressed on the cell surface where it exists in an equilibrium between a dimeric and monomeric form, the balance of which may be determined by factors including ligand binding. In response to an undefined stimulus, FL_{166} is internalised in association with the amino-terminal cleavage event producing E_{81} and P_{85}. This may occur either immediately before internalisation, possibly stimulating PTPσ internalisation or may be an intracellular event. A significant proportion of E_{81} is retained in an intracellular site. Separately, PTPσ is subject to carboxyl-terminal cleavage to generate E_{90} and P_{75}. This occurs at the cell surface and is associated with ectodomain shedding but is not alone sufficient for this process. It remains unclear whether molecules initially cleaved at one site are secondarily subject to cleavage at the other, although this is likely.

Lastly, it will be of interest to fully characterise the alternatively spliced isoforms and the expression pattern of chick LAR. Indeed, it would also be of value to complete the isoform specific expression pattern studies of chick PTPσ and PTPδ. At the current time, despite their multiple isoforms that may have distinct functional properties, studies generally only consider a single or at most two species. The chicken LAR clone 1 cDNA was submitted directly to Genbank as *Gallus gallus receptor protein tyrosine phosphatase LAR mRNA, partial cds* (Accession number pending).

Several future lines of work are suggested by this thesis. Firstly, the significance of PTPσ processing should not be underestimated. Therefore, PTPσ cleavage and dimerisation need further characterisation, with particular emphasis on the effects of ligand

PTPσ. It may be that simple assays such as formation of high molecular weight species may provide some insight into the effects of empirical manipulation on PTPσ dimerisation or conformation. Secondly, it will be important to identify the substrates and pathways with which PTPσ interacts. This will also facilitate the study of the effects of dimerisation or ligand binding on PTPσ function. Current approaches modelled on the functional behaviour of tyrosine kinases have only been of limited value. It may be that alternative approaches are needed to identify the function of PTPσ.

The work described in this thesis was presented in a recent publication that addressed one of the fundamental issues in the RPTP field – how ligand binding is affected by RPTP dimerisation. This paper included the disulphide crosslinking and co-immunoprecipitation analysis of PTPσ dimerisation presented in this thesis. In addition, data from other researchers was used to refine the dimer interface to the transmembrane domain and to demonstrate the effects of dimerisation on the PTPσ-ligand interaction. In this paper, it was reported for the first time that PTPσ exists in a dimeric state within cells. In addition, it was shown that only dimeric PTPσ is able to bind the heterophilic basement membrane and muscle ligands, and that the ligand binding specificities of the short PTPσ1 and longer PTPσ2 isoforms are not due to a straightforward relationship between ligand binding and domain structure. Instead, it was proposed that the different patterns of type II RPTP ligand binding might reflect different rotational conformations of the dimerised extracellular domains (Lee et al., 2006a). This model of rotational conformers was first proposed in a previous PhD thesis (Chilton, 2000).

Appendix 1: cLAR DNA sequences

LAR clone 1 – p289

LAR clone 2 – p296

The two sequence variations between LAR clone 1 (bp 2054, 2452) and LAR clone 2 (bp 1923, 2321) are highlighted in red. The region of the LAR clones derived from the oligonucleotide primers is *in italics and underlined.*

5' EST clone – p302

Sequence derived from chick EST (ChEST35h5) in publicly available UMIST/BBSRC database to extend 5' end of LAR clone 1 to include initiation codon, which is highlighted in red.

3' EST clone - p302

Sequence derived from chick EST (ChEST371m20) in publicly available UMIST/BBSRC database to extend 3' end of LAR clone 1 to include stop codon, which is highlighted in red.

LAR Clone 1

```
         10         20         30         40         50
....|....| ....|....| ....|....| ....|....| ....|....|
GTTTGATGAT GGGTCGGGAT CTGTTCTGCG GATACAGCCG CTGCGTGTCC
         60         70         80         90        100
....|....| ....|....| ....|....| ....|....| ....|....|
ATCGAGACGA AGCCATCTAC GAGTGCACGG CCACCAACAG CGTTGGGGAG
        110        120        130        140        150
....|....| ....|....| ....|....| ....|....| ....|....|
ATAAACACAA GTGCCAAATT AACAGTGTTA GAAGAAGACC ACCTCCCAGC
        160        170        180        190        200
....|....| ....|....| ....|....| ....|....| ....|....|
TGGCTTCCCA ACCATTGATA TGGGCCCGCA GCTGAAGGTG GTGGAGAAGG
        210        220        230        240        250
....|....| ....|....| ....|....| ....|....| ....|....|
CCCGCACAGC CACGATGCTC TGTGCTGCCA GTGGTAACCC CGATCCCGAA
        260        270        280        290        300
....|....| ....|....| ....|....| ....|....| ....|....|
ATCTCCTGGT TCAAGGACTT CCTGCCTGTt GACACTGCAA CTAGCAATGG
        310        320        330        340        350
....|....| ....|....| ....|....| ....|....| ....|....|
GAGAATCAAG CAGCTCCGCT CGGGAGCTCT GCAAATAGAG AACAGCGAGG
        360        370        380        390        400
....|....| ....|....| ....|....| ....|....| ....|....|
AGTCGGACCA AGGCAAATAT GAATGTGTTG CAACCAACAG CGCAGGAACC
        410        420        430        440        450
....|....| ....|....| ....|....| ....|....| ....|....|
CGTTATTCTG CCCCGGCGAA CCTGTATGTT CGAGTGCGGC GTGTTGCTCC
        460        470        480        490        500
....|....| ....|....| ....|....| ....|....| ....|....|
TCGATTCTCA ATCCCTCCGA GCAACCACGA GGTGATGCCT GGAGGGAGCG
        510        520        530        540        550
....|....| ....|....| ....|....| ....|....| ....|....|
TGAACCTCAC GTgCGTgGCA GTAGGTGCCC CCATGCCCTA CGTGAAGTGG
        560        570        580        590        600
....|....| ....|....| ....|....| ....|....| ....|....|
ATGGCTGGTG TGGAGGAGCT GACCAAAGAG GATGAGATGC CCGTCGGCCG
        610        620        630        640        650
....|....| ....|....| ....|....| ....|....| ....|....|
GAACgTGCTg GAGCTGAACA ACATCATGCA GTCCGCCGAC TACACCTGTG
        660        670        680        690        700
....|....| ....|....| ....|....| ....|....| ....|....|
TGGCCATCTC TTCCCTCGGC ATGATCGAAG CAACGGCCCA GATCACTATC
        710        720        730        740        750
....|....| ....|....| ....|....| ....|....| ....|....|
AAAGCGCTGC CAAAACCTCC AACAGAGCCA TTAGTGACTG AAACGACAGC
        760        770        780        790        800
....|....| ....|....| ....|....| ....|....| ....|....|
TACCAGCGTG ACCCTCACCT GGGATTCAGG CAACTCCGAC CCCATTTCAT
```

LAR Clone 1 (ctd)

```
        810         820         830         840         850
....|....| ....|....| ....|....| ....|....| ....|....|
ATTACGTCAT CCAGTACAAG CCCAAATCCT TGGAGGGTCA GTTCCAGGAG
        860         870         880         890         900
....|....| ....|....| ....|....| ....|....| ....|....|
GTGGACGGTG TGGCGACAAC CCGTTATAGC ATAGGTGGGC TCAGCCCCTT
        910         920         930         940         950
....|....| ....|....| ....|....| ....|....| ....|....|
CTCGGAGTAC GAGTTCCGGG TCATTGCAGT CAATAACATT GGCAGGGGCC
        960         970         980         990        1000
....|....| ....|....| ....|....| ....|....| ....|....|
CCCCCAGCGA GCTGGTCGAG GCCCGGACAG GAGAGCAAGC TCCTTCAAGT
       1010        1020        1030        1040        1050
....|....| ....|....| ....|....| ....|....| ....|....|
CCACCCCTCA AAGTCCAGGC GCGGATGCTG AGTGCCAGCA CCATGCTGGT
       1060        1070        1080        1090        1100
....|....| ....|....| ....|....| ....|....| ....|....|
CCAGTGGGAG CAGCCAGAGG AGCCAAATGG ACAGATCCGA GGGTACCGCG
       1110        1120        1130        1140        1150
....|....| ....|....| ....|....| ....|....| ....|....|
TTTACTACAC CACCGACTCG CATCTGCCTT CGAGCATGTG GCAAAAACAC
       1160        1170        1180        1190        1200
....|....| ....|....| ....|....| ....|....| ....|....|
AACACCGACG ACAGCCATCT CACCACTGTT GGGAGCCTCA TCACAGGGAC
       1210        1220        1230        1240        1250
....|....| ....|....| ....|....| ....|....| ....|....|
CACATACAGC ATCCGTGTCC TGGCCTTCAC TTCGGTGGGC GACGGGCCGC
       1260        1270        1280        1290        1300
....|....| ....|....| ....|....| ....|....| ....|....|
CCTCGGACAT CATCCAGGTC AAAACGCAGC AAGGAGTTCC TGCCCAGCCA
       1310        1320        1330        1340        1350
....|....| ....|....| ....|....| ....|....| ....|....|
GCTGACTTCC AGGCAGAGGC AGAGTCTGAT ACCCGCATCC TGCTGACGTG
       1360        1370        1380        1390        1400
....|....| ....|....| ....|....| ....|....| ....|....|
GCTGCCTGCC TCTCAGGAAC GCATTACCAA ATATGAGCTG CTGTACTGGG
       1410        1420        1430        1440        1450
....|....| ....|....| ....|....| ....|....| ....|....|
AAGGAGAGGA CGGTGTTCAG CAAAAAGTGG AGTTTGACCC AACCTCCTCG
       1460        1470        1480        1490        1500
....|....| ....|....| ....|....| ....|....| ....|....|
TATGCTGTGG AAGGTCTCGA ACCAGACACG CTGTACAAGT TCCGGCTGGG
       1510        1520        1530        1540        1550
....|....| ....|....| ....|....| ....|....| ....|....|
TGCCCACTCT GAGCTGGGGG TAGGAGTCTA CACCCCAACT GTGGAAGCCA
       1560        1570        1580        1590        1600
....|....| ....|....| ....|....| ....|....| ....|....|
GAACTGCCCA ATCCACCCCT TCTGCCCCAC CCCAAGAAGT CGAGTGTGTA
       1610        1620        1630        1640        1650
....|....| ....|....| ....|....| ....|....| ....|....|
AGCACCAGCT CCACTACCAT CCGGGTAAGT TGGGTGCCAC CGCCTGCACA
```

LAR Clone 1 (ctd)

```
        1660       1670       1680       1690       1700
....|....| ....|....| ....|....| ....|....| ....|....|
GAGCCGCAAT GGGGTCATCA CCCAGTACTC CATTGCGTAC CAGGCAGTGG
        1710       1720       1730       1740       1750
....|....| ....|....| ....|....| ....|....| ....|....|
AAGGAGATGA CAGCACCAAG CATGTGGTGG ATGGCATTGG ACGGGAGCAC
        1760       1770       1780       1790       1800
....|....| ....|....| ....|....| ....|....| ....|....|
TCCAGCTGGG AGATCAAGGA CCTGGAGAAA TGGACCGAGT ACAAGGTGTG
        1810       1820       1830       1840       1850
....|....| ....|....| ....|....| ....|....| ....|....|
GGTAAGGGCT CACACCGATG TGGGGCCAGG ACCGGAGAGC ATCCCCGTGC
        1860       1870       1880       1890       1900
....|....| ....|....| ....|....| ....|....| ....|....|
ACGTGCGCAC TGATGAGGAT GTGCCCAGTG CCCCACCACG AAAGGTGGAG
        1910       1920       1930       1940       1950
....|....| ....|....| ....|....| ....|....| ....|....|
GTGGAGTCCG TGAATTCAAC AGCCATCCGG GTGAGCTGGA AGCTGCCCAT
        1960       1970       1980       1990       2000
....|....| ....|....| ....|....| ....|....| ....|....|
CTCCAACAAG CAGCATGGGC AGATCCGGGG CTACCAGGTG ACCTACGTGA
        2010       2020       2030       2040       2050
....|....| ....|....| ....|....| ....|....| ....|....|
AGCTGGAAAA CAACGAGCCC CGCGGGCAGC CCGTCATCAA GGACGTCATG
        2060       2070       2080       2090       2100
....|....| ....|....| ....|....| ....|....| ....|....|
CTGTCGGAAG CACAGTGGAG AGCTGAGGAC TCCGTCGACC ATGAGACGGT
        2110       2120       2130       2140       2150
....|....| ....|....| ....|....| ....|....| ....|....|
CATTGGAGGC CTGCTGCCGG AAACCACCTA CTCCGTCACA GTCGCTGCCT
        2160       2170       2180       2190       2200
....|....| ....|....| ....|....| ....|....| ....|....|
ACACAACCAA GGGAGATGGT GCCCGCAGCA AGCCCAAGGT CATCACCACC
        2210       2220       2230       2240       2250
....|....| ....|....| ....|....| ....|....| ....|....|
ACAGGGGCAG TCCCGGGGAA GCCCACCATG ATGATTAGCA CGACAGCCAT
        2260       2270       2280       2290       2300
....|....| ....|....| ....|....| ....|....| ....|....|
GAACACAGCC CTCATCCAGT GGCACCCACC CAAGGAGATG GTAGGGGAGC
        2310       2320       2330       2340       2350
....|....| ....|....| ....|....| ....|....| ....|....|
TGCTGGGTTA CCGGCTGCAG TACAGACGTC TTGATGAgGA AAAGATGAAC
        2360       2370       2380       2390       2400
....|....| ....|....| ....|....| ....|....| ....|....|
ACGATAGACT TCGGGAAGAG AGACCATCAC TACACTGTGA CCAACTTGCA
        2410       2420       2430       2440       2450
....|....| ....|....| ....|....| ....|....| ....|....|
CAAGGGGGCC ACGTACCTCT TCAGACTGTC TGCCAAGAAC AGAGCTGGTC
        2460       2470       2480       2490       2500
....|....| ....|....| ....|....| ....|....| ....|....|
CAGGAGAGGA GTTTGAGAAG GAGATCACTA CCGCAGAAGA CGTGCCGAGT
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LAR Clone 1 (ctd)

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        2510       2520       2530       2540       2550
....|....| ....|....| ....|....| ....|....| ....|....|
GGATTCCCAC AGAACCTGCG CGTGGTGGGC CTCACCACTT CCACCACAGA
        2560       2570       2580       2590       2600
....|....| ....|....| ....|....| ....|....| ....|....|
GGTTGCATGG GACCCCCCAG TCTTGGCGGA AAGAAACGGC AAGATTGTCA
        2610       2620       2630       2640       2650
....|....| ....|....| ....|....| ....|....| ....|....|
ACTACACAGT GGTATACAGA GACATAAATA GCCAGCAGGA CCTGGTTAAC
        2660       2670       2680       2690       2700
....|....| ....|....| ....|....| ....|....| ....|....|
ATCACCAAGG ACACAAGTAT CACTTTGACG AATTTGAAGC CTGATACCAC
        2710       2720       2730       2740       2750
....|....| ....|....| ....|....| ....|....| ....|....|
TTATGACATC AAAGTGAGGG CCCGCACCAA TAAGGGGGTT GGGCCGCTCA
        2760       2770       2780       2790       2800
....|....| ....|....| ....|....| ....|....| ....|....|
GCCCCAGCAT CCAGTCCCGG ACCATGCCTG TTGAGCAAGT GTTTGCTAAG
        2810       2820       2830       2840       2850
....|....| ....|....| ....|....| ....|....| ....|....|
AACTTCCGTG TCAACGCCGT CATGAAAACA TCCGTGTTGC TGAGCTGGGA
        2860       2870       2880       2890       2900
....|....| ....|....| ....|....| ....|....| ....|....|
AGTGCCTGAC TCCTATAAAT CTGCAGTGCC TTTTAAGATT CTGTACAACA
        2910       2920       2930       2940       2950
....|....| ....|....| ....|....| ....|....| ....|....|
GCCAGAGTGT GGAGGTGGAC GGCTACTCCA TGAAGAAGCT CATAAGTGAC
        2960       2970       2980       2990       3000
....|....| ....|....| ....|....| ....|....| ....|....|
CTCCAGCCAG ACACGGACTA CTCTTTTGTG CTCATGAACC GTGGGAACAG
        3010       3020       3030       3040       3050
....|....| ....|....| ....|....| ....|....| ....|....|
TGCTGGGGGG CTCCAGCACC TCGTCTCCAT CCGCACTGCG CCTGACGTCT
        3060       3070       3080       3090       3100
....|....| ....|....| ....|....| ....|....| ....|....|
TGCAAAGCAA ACCCATTGCC ACCAACAAGT ATATCCAGGA AGGAAAGTTC
        3110       3120       3130       3140       3150
....|....| ....|....| ....|....| ....|....| ....|....|
ACGCTTACCC TTCCCAAAGT GCAGACCACT GTGCCAGTTC GGTGGTACTA
        3160       3170       3180       3190       3200
....|....| ....|....| ....|....| ....|....| ....|....|
CATTGTGGTC GTGCCGGCAG AGCAGAGCCC CAGCAGCCCG ACAGCGCGGT
        3210       3220       3230       3240       3250
....|....| ....|....| ....|....| ....|....| ....|....|
GGCGGACGCC TGATGAGATG GAGCTGGACC AGCTGCTGGA GGCCATAAGC
        3260       3270       3280       3290       3300
....|....| ....|....| ....|....| ....|....| ....|....|
CAGGGCAGTC AGAGCAGGCG CCAGAGACGC CAAGCAGACA GACTCAAGCC
        3310       3320       3330       3340       3350
....|....| ....|....| ....|....| ....|....| ....|....|
CTACATTGCT GCCCAAGTGG ACGTGCTGCC CGAGACCTTC ACCCTGGGGG
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LAR Clone 1 (ctd)

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        3360       3370       3380       3390       3400
....|....| ....|....| ....|....| ....|....| ....|....|
ACGAGAAGAA CTACAAGGGT TTCTACAACA AGCCCCTCTC TCAGGACCTG
        3410       3420       3430       3440       3450
....|....| ....|....| ....|....| ....|....| ....|....|
AGCTACCGCT GCTTCGTGCT GGCCTCGCTG GAGGATGGGG ACACGAAGAG
        3460       3470       3480       3490       3500
....|....| ....|....| ....|....| ....|....| ....|....|
ATACGCAGCC AGCCCCTACT CAGATGAGAT TGTGATGGAA TTGGCTTCAG
        3510       3520       3530       3540       3550
....|....| ....|....| ....|....| ....|....| ....|....|
CAAAGCAGCA GGATGAGCCA GAGATGCTGT GGGTGATGGG ACCTGTCCTG
        3560       3570       3580       3590       3600
....|....| ....|....| ....|....| ....|....| ....|....|
GCTGTGATAT TAATCATCAT CATCGTCATC GCTATACTCC TCTTCAAAAG
        3610       3620       3630       3640       3650
....|....| ....|....| ....|....| ....|....| ....|....|
GAAAAGAGCA CACTCCCCCT CTTCCAAAGA TGAGCACTCT ATTGGCCTGA
        3660       3670       3680       3690       3700
....|....| ....|....| ....|....| ....|....| ....|....|
AGGACTCGCT CTTGGCCCAC TCCTCTGACC CGGTTGAGAT GAGGCGGCTC
        3710       3720       3730       3740       3750
....|....| ....|....| ....|....| ....|....| ....|....|
AACTACCAGA CTCCAGGCAT GCGGGACCAC CCGCCCATCC CCGTGACCGA
        3760       3770       3780       3790       3800
....|....| ....|....| ....|....| ....|....| ....|....|
CCTCGCCGAC AACATCGACC GGCTGAAGGC CAACGATGGC CTCAAGTTCT
        3810       3820       3830       3840       3850
....|....| ....|....| ....|....| ....|....| ....|....|
CCCAGGAGTA CGAGTCCATC GATCCCGGGC AGCAGTTCAC GTGGGAGAAC
        3860       3870       3880       3890       3900
....|....| ....|....| ....|....| ....|....| ....|....|
TCCAACCTGG AAGTAAACAA ACCGAAAAAC CGCTATGCAA ACGTGATCGC
        3910       3920       3930       3940       3950
....|....| ....|....| ....|....| ....|....| ....|....|
CTACGATCAC TCCCGCGTCA TCCTGACCTC CATCGATGGG GCCCCAGGCA
        3960       3970       3980       3990       4000
....|....| ....|....| ....|....| ....|....| ....|....|
GCGATTACAT CAACGCCAAT TACATCGACG GCTATAGGAA GCAGAACGCC
        4010       4020       4030       4040       4050
....|....| ....|....| ....|....| ....|....| ....|....|
TACATCGCGA CGCAGGGACC TCTGCCTGAA ACCCTCAGTG ACTTCTGGAG
        4060       4070       4080       4090       4100
....|....| ....|....| ....|....| ....|....| ....|....|
AATGGTGTGG GAGCAGAGAA CAGCAACGAT CGTCATGATG ACCAGGCTGG
        4110       4120       4130       4140       4150
....|....| ....|....| ....|....| ....|....| ....|....|
AGGAGAAGTC CAGGGTGAAG TGTGACCAGT ACTGGCCGAG CCGGGGTACA
        4160       4170       4180       4190       4200
....|....| ....|....| ....|....| ....|....| ....|....|
GAAACCTACG GGATGATCCA GGTTACACTG CTGGACACCG TTGAGCTGGC
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LAR Clone 1 (ctd)

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        4210        4220        4230        4240        4250
....|....| ....|....| ....|....| ....|....| ....|....|
GACCTACACA GTCCGAACCT TTGCCCTGTA CAAGAACGGC TCCAGCGAGA
        4260        4270        4280        4290        4300
....|....| ....|....| ....|....| ....|....| ....|....|
AGCGAGAGCT GCGACAGTTC CAGTTCATGG CATGGCCTGA TCACGGGGTC
        4310        4320        4330        4340        4350
....|....| ....|....| ....|....| ....|....| ....|....|
CCAGAATACC CCACCCCAAT CCTCGCTTTC CTGCGGCGGG TCAAGGCCTG
        4360        4370        4380        4390        4400
....|....| ....|....| ....|....| ....|....| ....|....|
CAACCCACCC GATGCTGGGC CCATGGTTGT CCATTGCAGT GCTGGCGTGG
        4410        4420        4430        4440        4450
....|....| ....|....| ....|....| ....|....| ....|....|
GCCGAACTGG TTGCTTCATT GTCATTGATG CCATGCTGGA GAGGATGAAG
        4460        4470        4480        4490        4500
....|....| ....|....| ....|....| ....|....| ....|....|
CATGAGAAGA CCGTGGACAT CTACGGGCAC GTGACGTGCA TGCGCTCGCA
        4510        4520        4530        4540        4550
....|....| ....|....| ....|....| ....|....| ....|....|
GCGCAACTAC ATGGTGCAGA CAGAGGACCA GTACATCTTC ATCCACGAGG
        4560        4570        4580        4590        4600
....|....| ....|....| ....|....| ....|....| ....|....|
CCCTGCTGGA GGCAGCCACG TGCGGCAATA CCGAGGTGCC TGCACGCAAC
        4610        4620        4630        4640        4650
....|....| ....|....| ....|....| ....|....| ....|....|
CTCTTCGCTC ACATCCAGAA ACTGACCCAG GTGCCTCCAG GAGAGAGCGT
        4660        4670        4680        4690        4700
....|....| ....|....| ....|....| ....|....| ....|....|
CACTGCGATG GAGCTGGAAT TCAAGCTGCT GGCCAACTCT AAAGCACATA
        4710        4720        4730        4740        4750
....|....| ....|....| ....|....| ....|....| ....|....|
CCTCACGTTT CATCAGTGCC AACCTCCCAT GCAACAAATT CAAGAACCGC
        4760        4770        4780        4790        4800
....|....| ....|....| ....|....| ....|....| ....|....|
CTCGTGAACA TCATGCCATA CGAGCTGACA AGAGTCTGCC TGCAGCCCAT
        4810        4820        4830        4840        4850
....|....| ....|....| ....|....| ....|....| ....|....|
CCGGGGTGTT GAGGGCTCTG ATTACATCAA TGCCAGCTTC ATTGATGGGT
        4860        4870        4880        4890        4900
....|....| ....|....| ....|....| ....|....| ....|....|
ACAGGCAGCA GAAGGCCTAC ATTGCCACGC AGGGACCACT GGCCGAGACC
        4910        4920        4930        4940        4950
....|....| ....|....| ....|....| ....|....| ....|....|
ACAGAAGATT TCTGGCGAAT GCTCTGGGAG CACAACTCCA CCATTGTGGT
        4960        4970        4980        4990        5000
....|....| ....|....| ....|....| ....|....| ....|....|
GATGTTGACA AAGCTGCGTG AGATGGGCCG GGAGAAGTGC CATCAGTACT
        5010        5020        5030        5040        5050
....|....| ....|....| ....|....| ....|....| ....|....|
GGCCTGCGGA GCGCTCTGCC CGTTACCAGT ACTTCGTGGT GGACCCCATG
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LAR Clone 1 (ctd)

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        5060        5070        5080        5090        5100
....|....| ....|....| ....|....| ....|....| ....|....|
GCAGAGTACA ACATGCCGCA GTACATCCTC CGGGAGTTCA AAGTCACAGA
        5110        5120        5130        5140        5150
....|....| ....|....| ....|....| ....|....| ....|....|
TGCCAGGGAC GGCCAGTCAC GGACCATCCG CCAGTTCCAG TTCACAGACT
        5160        5170        5180        5190        5200
....|....| ....|....| ....|....| ....|....| ....|....|
GGCCTGAGCA GGGAGTGCCA AAGACGGGAG AGGGCTTCAT CGACTTCATT
        5210        5220        5230        5240        5250
....|....| ....|....| ....|....| ....|....| ....|....|
GGGCAGGTGC ACAAGACCAA GGAGCAGTTT GGTCAGGACG GGCCCATCAC
        5260        5270        5280        5290        5300
....|....| ....|....| ....|....| ....|....| ....|....|
GGTGCACTGC AGCGCTGGGG TCGGGCGCAC GGGCGTGTTC ATCACACTGA
        5310        5320        5330        5340        5350
....|....| ....|....| ....|....| ....|....| ....|....|
GCATCGTGCT GGAGAGGATG CGCTACGAGG GGGTGGTGGA CATGTTCCAG
        5360        5370        5380
....|....| ....|....| ....|....| .
ACGGTGAAGA CTTTGCGGAC GCAGCGGCCGG
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LAR Clone 2

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         10         20         30         40         50
....|....| ....|....| ....|....| ....|....| ....|....|
AAGAAGACCA CCTCCCAGCT GGCTTCCCAA CCATTGATAT GGGCCCGCAG
         60         70         80         90        100
....|....| ....|....| ....|....| ....|....| ....|....|
CTGAAGGTGG TGGAGAAGGC CCGCACAGCC ACGATGCTCT GTGCTGCCAG
        110        120        130        140        150
....|....| ....|....| ....|....| ....|....| ....|....|
TGGTAACCCC GATCCCGAAA TCTCCTGGTT CAAGGACTTC CTGCCTGTTG
        160        170        180        190        200
....|....| ....|....| ....|....| ....|....| ....|....|
ACACTGCAAC TAGCAATGGG AGAATCAAGC AGCTCCGCTC GGGAGCTCTG
        210        220        230        240        250
....|....| ....|....| ....|....| ....|....| ....|....|
CAAATAGAGA ACAGCGAGGA GTCGGACCAA GGCAAATATG AATGTGTTGC
        260        270        280        290        300
....|....| ....|....| ....|....| ....|....| ....|....|
AACCAACAGC GCAGGAACCC GTTATTCTGC CCCGGCGAAC CTGTATGTTC
        310        320        330        340        350
....|....| ....|....| ....|....| ....|....| ....|....|
GAGTGCGGCG TGTTGCTCCT CGATTCTCAA TCCCTCCGAG CAACCACGAG
        360        370        380        390        400
....|....| ....|....| ....|....| ....|....| ....|....|
GTGATGCCTG GAGGGAGCGT GAACCTCACG TGCGTGGCAG TAGGTGCCCC
        410        420        430        440        450
....|....| ....|....| ....|....| ....|....| ....|....|
CATGCCCTAC GTGAAGTGGA TGGCTGGTGT GGAGGAGCTG ACCAAAGAGG
        460        470        480        490        500
....|....| ....|....| ....|....| ....|....| ....|....|
ATGAGATGCC CGTCGGCCGG AACGTGCTGG AGCTGAACAA CATCATGCAG
        510        520        530        540        550
....|....| ....|....| ....|....| ....|....| ....|....|
TCCGCCGACT ACACCTGTGT GGCCATCTCT TCCCTCGGCA TGATCGAAGC
        560        570        580        590        600
....|....| ....|....| ....|....| ....|....| ....|....|
AACGGCCCAG ATCACTATCA AAGCGCTGCC AAAACCTCCA ACAGAGCCAT
        610        620        630        640        650
....|....| ....|....| ....|....| ....|....| ....|....|
TAGTGACTGA AACGACAGCT ACCAGCGTGA CCCTCACCTG GGATTCAGGC
        660        670        680        690        700
....|....| ....|....| ....|....| ....|....| ....|....|
AACTCCGACC CCATTTCATA TTACGTCATC CAGTACAAGC CCAAATCCTT
        710        720        730        740        750
....|....| ....|....| ....|....| ....|....| ....|....|
GGAGGGTCAG TTCCAGGAGG TGGACGGTGT GGCGACAACC CGTTATAGCA
        760        770        780        790        800
....|....| ....|....| ....|....| ....|....| ....|....|
TAGGTGGGCT CAGCCCCTTC TCGGAGTACG AGTTCCGGGT CATTGCAGTC
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LAR Clone 2 (Ctd)

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        810         820         830         840         850
....|....| ....|....| ....|....| ....|....| ....|....|
AATAACATTG GCAGGGGCCC CCCCAGCGAG CTGGTCGAGG CCCGGACAGG
        860         870         880         890         900
....|....| ....|....| ....|....| ....|....| ....|....|
AGAGCAAGCT CCTTCAAGTC CACCCCTCAA AGTCCAGGCG CGGATGCTGA
        910         920         930         940         950
....|....| ....|....| ....|....| ....|....| ....|....|
GTGCCAGCAC CATGCTGGTC CAGTGGGAGC AGCCAGAGGA GCCAAATGGA
        960         970         980         990        1000
....|....| ....|....| ....|....| ....|....| ....|....|
CAGATCCGAG GGTACCGCGT TTACTACACC ACCGACTCGC ATCTGCCTTC
       1010        1020        1030        1040        1050
....|....| ....|....| ....|....| ....|....| ....|....|
GAGCATGTGG CAAAAACACA ACACCGACGA CAGCCATCTC ACCACTGTTG
       1060        1070        1080        1090        1100
....|....| ....|....| ....|....| ....|....| ....|....|
GGAGCCTCAT CACAGGGACC ACATACAGCA TCCGTGTCCT GGCCTTCACT
       1110        1120        1130        1140        1150
....|....| ....|....| ....|....| ....|....| ....|....|
TCGGTGGGCG ACGGGCCGCC CTCGGACATC ATCCAGGTCA AAACGCAGCA
       1160        1170        1180        1190        1200
....|....| ....|....| ....|....| ....|....| ....|....|
AGGAGTTCCT GCCCAGCCAG CTGACTTCCA GGCAGAGGCA GAGTCTGATA
       1210        1220        1230        1240        1250
....|....| ....|....| ....|....| ....|....| ....|....|
CCCGCATCCT GCTGACGTGG CTGCCTGCCT CTCAGGAACG CATTACCAAA
       1260        1270        1280        1290        1300
....|....| ....|....| ....|....| ....|....| ....|....|
TATGAGCTGC TGTACTGGGA AGGAGAGGAC GGTGTTCAGC AAAAAGTGGA
       1310        1320        1330        1340        1350
....|....| ....|....| ....|....| ....|....| ....|....|
GTTTGACCCA ACCTCCTCGT ATGCTGTGGA AGGTCTCGAA CCAGACACGC
       1360        1370        1380        1390        1400
....|....| ....|....| ....|....| ....|....| ....|....|
TGTACAAGTT CCGGCTGGGT GCCCACTCTG AGCTGGGGGT AGGAGTCTAC
       1410        1420        1430        1440        1450
....|....| ....|....| ....|....| ....|....| ....|....|
ACCCCAACTG TGGAAGCCAG AACTGCCCAA TCCACCCCTT CTGCCCCACC
       1460        1470        1480        1490        1500
....|....| ....|....| ....|....| ....|....| ....|....|
CCAAGAAGTC GAGTGTGTAA GCACCAGCTC CACTACCATC CGGGTAAGTT
       1510        1520        1530        1540        1550
....|....| ....|....| ....|....| ....|....| ....|....|
GGGTGCCACC GCCTGCACAG AGCCGCAATG GGGTCATCAC CCAGTACTCC
       1560        1570        1580        1590        1600
....|....| ....|....| ....|....| ....|....| ....|....|
ATTGCGTACC AGGCAGTGGA AGGAGATGAC AGCACCAAGC ATGTGGTGGA
       1610        1620        1630        1640        1650
....|....| ....|....| ....|....| ....|....| ....|....|
TGGCATTGGA CGGGAGCACT CCAGCTGGGA GATCAAGGAC CTGGAGAAAT
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LAR Clone 2 (Ctd)

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        1660       1670       1680       1690       1700
....|....| ....|....| ....|....| ....|....| ....|....|
GGACCGAGTA CAAGGTGTGG GTAAGGGCTC ACACCGATGT GGGGCCAGGA
        1710       1720       1730       1740       1750
....|....| ....|....| ....|....| ....|....| ....|....|
CCGGAGAGCA TCCCCGTGCA CGTGCGCACT GATGAGGATG TGCCCAGTGC
        1760       1770       1780       1790       1800
....|....| ....|....| ....|....| ....|....| ....|....|
CCCACCACGA AAGGTGGAGG TGGAGTCCGT GAATTCAACA GCCATCCGGG
        1810       1820       1830       1840       1850
....|....| ....|....| ....|....| ....|....| ....|....|
TGAGCTGGAA GCTGCCCATC TCCAACAAGC AGCATGGGCA GATCCGGGGC
        1860       1870       1880       1890       1900
....|....| ....|....| ....|....| ....|....| ....|....|
TACCAGGTGA CCTACGTGAA GCTGGAAAAC AACGAGCCCC GCGGGCAGCC
        1910       1920       1930       1940       1950
....|....| ....|....| ....|....| ....|....| ....|....|
CGTCATCAAG GACGTCATGC TGCCGGAAGC ACAGTGGAGA GCTGAGGACT
        1960       1970       1980       1990       2000
....|....| ....|....| ....|....| ....|....| ....|....|
CCGTCGACCA TGAGACGGTC ATTGGAGGCC TGCTGCCGGA AACCACCTAC
        2010       2020       2030       2040       2050
....|....| ....|....| ....|....| ....|....| ....|....|
TCCGTCACAG TCGCTGCCTA CACAACCAAG GGAGATGGTG CCCGCAGCAA
        2060       2070       2080       2090       2100
....|....| ....|....| ....|....| ....|....| ....|....|
GCCCAAGGTC ATCACCACCA CAGGGGCAGT CCCGGGGAAG CCCACCATGA
        2110       2120       2130       2140       2150
....|....| ....|....| ....|....| ....|....| ....|....|
TGATTAGCAC GACAGCCATG AACACAGCCC TCATCCAGTG GCACCCACCC
        2160       2170       2180       2190       2200
....|....| ....|....| ....|....| ....|....| ....|....|
AAGGAGATGG TAGGGGAGCT GCTGGGTTAC CGGCTGCAGT ACAGACGTCT
        2210       2220       2230       2240       2250
....|....| ....|....| ....|....| ....|....| ....|....|
TGATGAGGAA AAGATGAACA CGATAGACTT CGGGAAGAGA GACCATCACT
        2260       2270       2280       2290       2300
....|....| ....|....| ....|....| ....|....| ....|....|
ACACTGTGAC CAACTTGCAC AAGGGGGCCA CGTACCTCTT CAGACTGTCT
        2310       2320       2330       2340       2350
....|....| ....|....| ....|....| ....|....| ....|....|
GCCAAGAACA GAGCTGGTCC GGGAGAGGAG TTTGAGAAGG AGATCACTAC
        2360       2370       2380       2390       2400
....|....| ....|....| ....|....| ....|....| ....|....|
CGCAGAAGAC GTGCCGAGTG GATTCCCACA GAACCTGCGC GTGGTGGGCC
        2410       2420       2430       2440       2450
....|....| ....|....| ....|....| ....|....| ....|....|
TCACCACTTC CACCACAGAG GTTGCATGGG ACCCCCCAGT CTTGGCGGAA
        2460       2470       2480       2490       2500
....|....| ....|....| ....|....| ....|....| ....|....|
AGAAACGGCA AGATTGTCAA CTACACAGTG GTATACAGAG ACATAAATAG
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LAR Clone 2 (Ctd)

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        2510       2520       2530       2540       2550
....|....| ....|....| ....|....| ....|....| ....|....|
CCAGCAGGAC CTGGTTAACA TCACCAAGGA CACAAGTATC ACTTTGACGA
        2560       2570       2580       2590       2600
....|....| ....|....| ....|....| ....|....| ....|....|
ATTTGAAGCC TGATACCACT TATGACATCA AAGTGAGGGC CCGCACCAAT
        2610       2620       2630       2640       2650
....|....| ....|....| ....|....| ....|....| ....|....|
AAGGGGGTTG GGCCGCTCAG CCCCAGCATC CAGTCCCGGA CCATGCCTGT
        2660       2670       2680       2690       2700
....|....| ....|....| ....|....| ....|....| ....|....|
TGAGCAAGTG TTTGCTAAGA ACTTCCGTGT CAACGCCGTC ATGAAAACAT
        2710       2720       2730       2740       2750
....|....| ....|....| ....|....| ....|....| ....|....|
CCGTGTTGCT GAGCTGGGAA GTGCCTGACT CCTATAAATC TGCAGTGCCT
        2760       2770       2780       2790       2800
....|....| ....|....| ....|....| ....|....| ....|....|
TTTAAGATTC TGTACAACAG CCAGAGTGTG GAGGTGGACG GCTACTCCAT
        2810       2820       2830       2840       2850
....|....| ....|....| ....|....| ....|....| ....|....|
GAAGAAGCTC ATAAGTGACC TCCAGCCAGA CACGGACTAC TCTTTTGTGC
        2860       2870       2880       2890       2900
....|....| ....|....| ....|....| ....|....| ....|....|
TCATGAACCG TGGGAACAGT GCTGGGGGGC TCCAGCACCT CGTCTCCATC
        2910       2920       2930       2940       2950
....|....| ....|....| ....|....| ....|....| ....|....|
CGCACTGCGC CTGACGTCTT GCAAAGCAAA CCCATTGCCA CCAACAAGTA
        2960       2970       2980       2990       3000
....|....| ....|....| ....|....| ....|....| ....|....|
TATCCAGGAA GGAAAGTTCA CGCTTACCCT TCCCAAAGTG CAGACCACTG
        3010       3020       3030       3040       3050
....|....| ....|....| ....|....| ....|....| ....|....|
TGCCAGTTCG GTGGTACTAC ATTGTGGTCG TGCCGGCAGA GCAGAACCCC
        3060       3070       3080       3090       3100
....|....| ....|....| ....|....| ....|....| ....|....|
AGCAGCCCGA CAGCGCGGTG GCGGACGCCT GATGAGATGG AGCTGGACCA
        3110       3120       3130       3140       3150
....|....| ....|....| ....|....| ....|....| ....|....|
GCTGCTGGAG GCCATAAGCC AGGGCAGTCA GAGCAGGCGC CAGAGACGCC
        3160       3170       3180       3190       3200
....|....| ....|....| ....|....| ....|....| ....|....|
AAGCAGACAG ACTCAAGCCC TACATTGCTG CCCAAGTGGA CGTGCTGCCC
        3210       3220       3230       3240       3250
....|....| ....|....| ....|....| ....|....| ....|....|
GAGACCTTCA CCCTGGGGGA CGAGAAGAAC TACAAGGGTT TCTACAACAA
        3260       3270       3280       3290       3300
....|....| ....|....| ....|....| ....|....| ....|....|
GCCCCTCTCT CAGGACCTGA GCTACCGCTG CTTCGTGCTG GCCTCGCTGG
        3310       3320       3330       3340       3350
....|....| ....|....| ....|....| ....|....| ....|....|
AGGATGGGGA CACGAAGAGA TACGCAGCCA GCCCCTACTC AGATGAGATT
```

LAR Clone 2 (Ctd)

```
        3360       3370       3380       3390       3400
....|....| ....|....| ....|....| ....|....| ....|....|
GTGATGGAAT TGGCTTCAGC AAAGCAGCAG GATGAGCCAG AGATGCTGTG
        3410       3420       3430       3440       3450
....|....| ....|....| ....|....| ....|....| ....|....|
GGTGATGGGA CCTGTCCTGG CTGTGATATT AATCATCATC ATCGTCATCG
        3460       3470       3480       3490       3500
....|....| ....|....| ....|....| ....|....| ....|....|
CTATACTCCT CTTCAAAAGG AAAAGAGCAC ACTCCCCCTC TTCCAAAGAT
        3510       3520       3530       3540       3550
....|....| ....|....| ....|....| ....|....| ....|....|
GAGCACTCTA TTGGCCTGAA GGACTCGCTC TTGGCCCACT CCTCTGACCC
        3560       3570       3580       3590       3600
....|....| ....|....| ....|....| ....|....| ....|....|
GGTTGAGATG AGGCGGCTCA ACTACCAGAC TCCAGGCATG CGGGACCACC
        3610       3620       3630       3640       3650
....|....| ....|....| ....|....| ....|....| ....|....|
CGCCCATCCC CGTGACCGAC CTCGCCGACA ACATCGACCG GCTGAAGGCC
        3660       3670       3680       3690       3700
....|....| ....|....| ....|....| ....|....| ....|....|
AACGATGGCC TCAAGTTCTC CCAGGAGTAC GAGTCCATCG ATCCCGGGCA
        3710       3720       3730       3740       3750
....|....| ....|....| ....|....| ....|....| ....|....|
GCAGTTCACG TGGGAGAACT CCAACCTGGA AGTAAACAAA CCGAAAAACC
        3760       3770       3780       3790       3800
....|....| ....|....| ....|....| ....|....| ....|....|
GCTATGCAAA CGTGATCGCC TACGATCACT CCCGCGTCAT CCTGACCTCC
        3810       3820       3830       3840       3850
....|....| ....|....| ....|....| ....|....| ....|....|
ATCGATGGGG CCCCAGGCAG CGATTACATC AACGCCAATT ACATCGACGG
        3860       3870       3880       3890       3900
....|....| ....|....| ....|....| ....|....| ....|....|
CTATAGGAAG CAGAACGCCT ACATCGCGAC GCAGGGACCT CTGCCTGAAA
        3910       3920       3930       3940       3950
....|....| ....|....| ....|....| ....|....| ....|....|
CCCTCAGTGA CTTCTGGAGA ATGGTGTGGG AGCAGAGAAC AGCAACGATC
        3960       3970       3980       3990       4000
....|....| ....|....| ....|....| ....|....| ....|....|
GTCATGATGA CCAGGCTGGA GGAGAAGTCC AGGGTGAAGT GTGACCAGTA
        4010       4020       4030       4040       4050
....|....| ....|....| ....|....| ....|....| ....|....|
CTGGCCGAGC CGGGGTACAG AAACCTACGG GATGATCCAG GTTACACTGC
        4060       4070       4080       4090       4100
....|....| ....|....| ....|....| ....|....| ....|....|
TGGACACCGT TGAGCTGGCG ACCTACACAG TCCGAACCTT TGCCCTGTAC
        4110       4120       4130       4140       4150
....|....| ....|....| ....|....| ....|....| ....|....|
AAGAACGGCT CCAGCGAGAA GCGAGAGCTG CGACAGTTCC AGTTCATGGC
        4160       4170       4180       4190       4200
....|....| ....|....| ....|....| ....|....| ....|....|
ATGGCCTGAT CACGGGGTCC CAGAATACCC CACCCCAATC CTCGCTTTCC
```

LAR Clone 2 (Ctd)

```
        4210        4220        4230        4240        4250
....|....| ....|....| ....|....| ....|....| ....|....|
TGCGGCGGGT CAAGGCCTGC AACCCACCCG ATGCTGGGCC CATGGTTGTC
        4260        4270        4280        4290        4300
....|....| ....|....| ....|....| ....|....| ....|....|
CATTGCAGTG CTGGCGTGGG CCGAACTGGT TGCTTCATTG TCATTGATGC
        4310        4320        4330        4340        4350
....|....| ....|....| ....|....| ....|....| ....|....|
CATGCTGGAG AGGATGAAGC ATGAGAAGAC CGTGGACATC TACGGGCACG
        4360        4370        4380        4390        4400
....|....| ....|....| ....|....| ....|....| ....|....|
TGACGTGCAT GCGCTCGCAG CGCAACTACA TGGTGCAGAC AGAGGACCAG
        4410        4420        4430        4440        4450
....|....| ....|....| ....|....| ....|....| ....|....|
TACATCTTCA TCCACGAGGC CCTGCTGGAG GCAGCCACGT GCGGCAATAC
        4460        4470        4480        4490        4500
....|....| ....|....| ....|....| ....|....| ....|....|
CGAGGTGCCT GCACGCAACC TCTTCGCTCA CATCCAGAAA CTGACCCAGG
        4510        4520        4530        4540        4550
....|....| ....|....| ....|....| ....|....| ....|....|
TGCCTCCAGG AGAGAGCGTC ACTGCGATGG AGCTGGAATT CAAGCTGCTG
        4560        4570        4580        4590        4600
....|....| ....|....| ....|....| ....|....| ....|....|
GCCAACTCTA AAGCACATAC CTCACGTTTC ATCAGTGCCA ACCTCCCATG
        4610        4620        4630        4640        4650
....|....| ....|....| ....|....| ....|....| ....|....|
CAACAAATTC AAGAACCGCC TCGTGAACAT CATGCCATAC GAGCTGACAA
        4660        4670        4680        4690        4700
....|....| ....|....| ....|....| ....|....| ....|....|
GAGTCTGCCT GCAGCCCATC CGGGGTGTTG AGGGCTCTGA TTACATCAAT
        4710        4720        4730        4740        4750
....|....| ....|....| ....|....| ....|....| ....|....|
GCCAGCTTCA TTGATGGGTA CAGGCAGCAG AAGGCCTACA TTGCCACGCA
        4760        4770        4780        4790        4800
....|....| ....|....| ....|....| ....|....| ....|....|
GGGACCACTG GCCGAGACCA CAGAAGATTT CTGGCGAATG CTCTGGGAGC
        4810        4820        4830        4840        4850
....|....| ....|....| ....|....| ....|....| ....|....|
ACAACTCCAC CATTGTGGTG ATGTTGACAA AGCTGCGTGA GATGGGCCGG
        4860        4870        4880        4890        4900
....|....| ....|....| ....|....| ....|....| ....|....|
GAGAAGTGCC ATCAGTACTG GCCTGCGGAG CGCTCTGCCC GTTACCAGTA
        4910        4920        4930        4940        4950
....|....| ....|....| ....|....| ....|....| ....|....|
CTTCGTGGTG GACCCCATGG CAGAGTACAA CATGCCGCAG TACATCCTCC
        4960        4970        4980        4990        5000
....|....| ....|....| ....|....| ....|....| ....|....|
GGGAGTTCAA AGTCACAGAT GCCAGGGACG GCCAGTCACG GACCATCCGC
        5010        5020        5030        5040        5050
....|....| ....|....| ....|....| ....|....| ....|....|
CAGTTCCAGT TCACAGACTG GCCTGAGCAG GGAGTGCCAA AGACGGGAGA
```

LAR Clone 2 (Ctd)

```
        5060        5070        5080        5090        5100
....|....| ....|....| ....|....| ....|....| ....|....|
GGGCTTCATC GACTTCATTG GGCAGGTGCA CAAGACCAAG GAGCAGTTTG
        5110        5120        5130        5140        5150
....|....| ....|....| ....|....| ....|....| ....|....|
GTCAGGACGG GCCCATCACG GTGCACTGCA GCGCTGGGGT TGGGCGCACG
        5160        5170        5180        5190        5200
....|....| ....|....| ....|....| ....|....| ....|....|
GGCGTGTTCA TCACACTGAG CATCGTGCTG GAGAGGATGC GCTACGAGGG
        5210        5220        5230        5240
....|....| ....|....| ....|....| ....|....| ...
GGTGGTGGAC ATGTTCCAGA CGGTGAAGAC TTTGCGGACG CAG
```

5' chicken LAR EST (ChEST35h5) sequence

```
....|....| ....|....| ....|....| ....|....| ....|....|
         10          20          30          40          50
GTAACGCTGG ATGTGCTGAC CACTTGCCTC AGACTGAAGA GGACTTGCTC
....|....| ....|....| ....|....| ....|....| ....|....|
         60          70          80          90          100
TGCTTTGGAA TATGGTGCCT AACTTCTGTG TCTCAGTACT CACTGTGCCA
....|....| ....|....| ....|....| ....|....| ....|....|
         110         120         130         140         150
GCAGTCATTC TGTGCTGCTT GATGCTGCCT GATCCTGCTA AAGCAGGAGC
....|....| ....|....| ....|....| ....|....| ....|....|
         160         170         180         190         200
CGAAAGCAAA CCAACATTTA TGAAAGCTCC TGAAGATCAG ATTGGGATTT
....|....| ....|....| ....|....| ....|....| ....|....|
         210         220         230         240         250
CGGGAGGAGT GGCCTCTTTT GTGTGTCAGG CAACAGGAGA GCCCAAGCCT
CGAATCACGT GGATGAAGAA GGGGAAGAAA GTCAGTTCTC AGCGGTTTGA
....|....| ....|....| ....|....| ....|....| ....|....|
         260
GGTGATCGAG
```

3' chicken LAR EST (ChEST371m20) sequence

```
....|....| ....|....| ....|....| ....|....| ....|....|
         10          20          30          40          50
GCCATGGTGC AGACAGAGGA CCAGTACCAG CTGTGCTACA GAGCGGCACT
....|....| ....|....| ....|....| ....|....| ....|....|
         60          70          80          90          100
GGAGTATCTC GGCAGCTTTG ACCACTATGC AACGTAACTA GTGCTTTCCT
.

C
```

Appendix 2: Plasmid vector maps

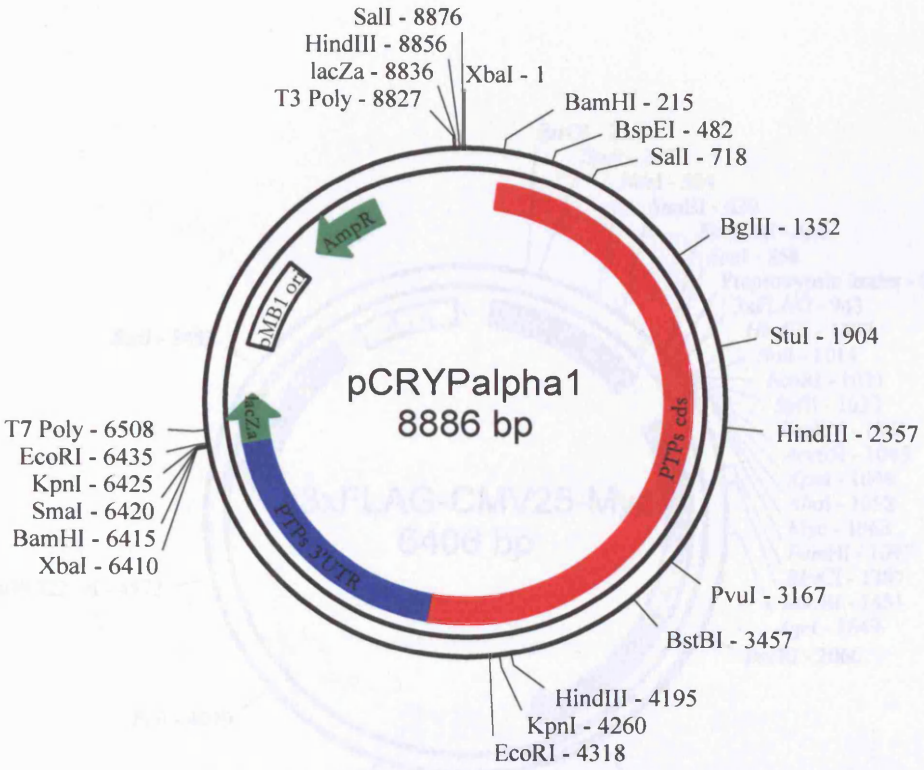
Details of vector backbone structure can be obtained from the original supplier/creator (2.3). Vector maps were generated using the freeware program pDRAW32 (http://www.acaclone.com/). The following colour legend was used:

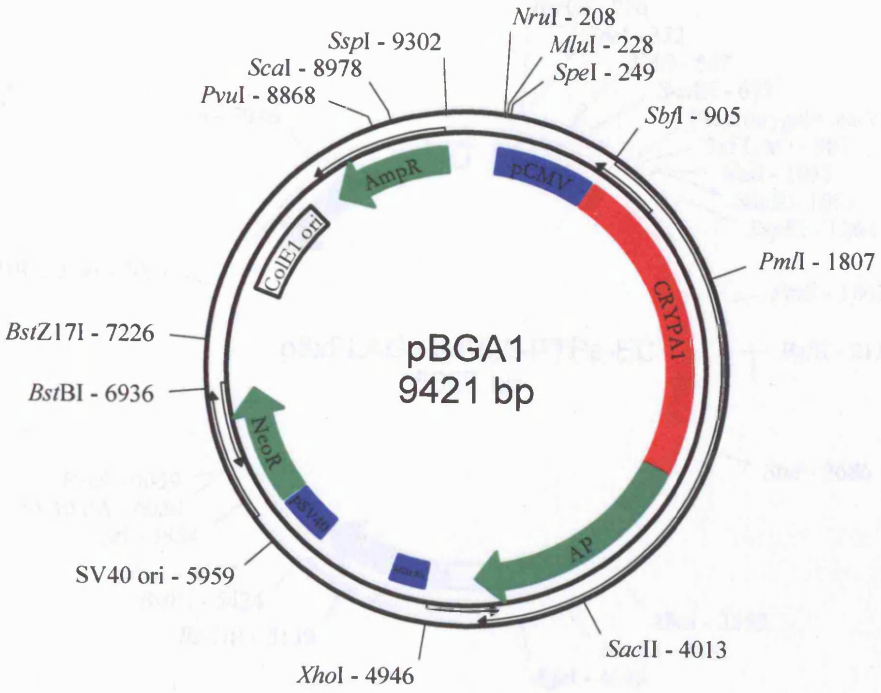
Red – cDNA inserts.

Green – Other open reading frames (Arrow indicates orientation).

Blue – Promoter, polyA signal or untranslated region.

Open boxes – Plasmid origins of replication.

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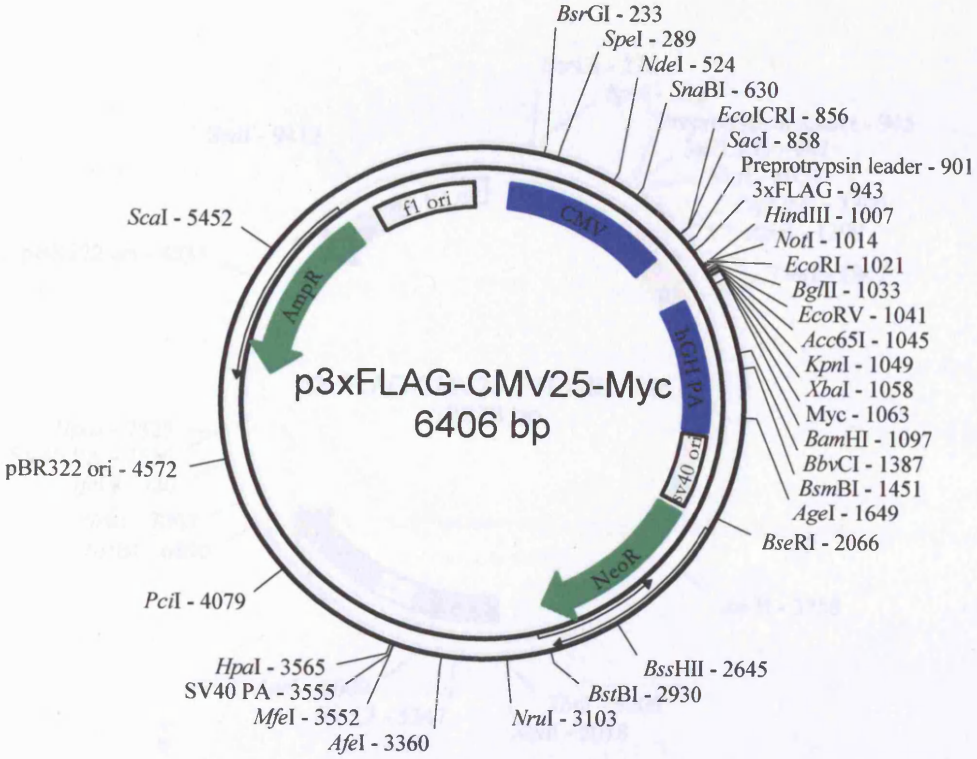
MluI - 228
SpeI - 249
SbfI - 905
PmlI - 1807
SacII - 4013
XhoI - 4946
SV40 ori - 5959
BstBI - 6936
BstZ17I - 7226
SspI - 9302
ScaI - 8978
PvuI - 8868
AmpR
pCMV
CRYPA1
AP
NeoR
ColE1 ori
pBGA1
9421 bp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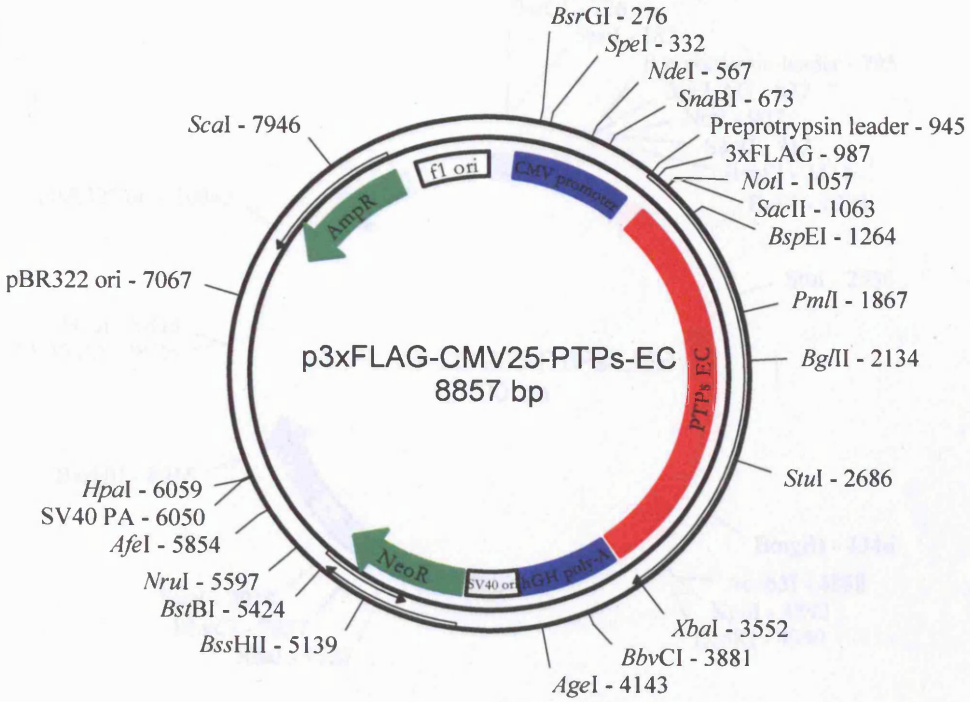
SpeI - 289
NdeI - 524
SnaBI - 630
EcoICRI - 856
SacI - 858
Preprotrypsin leader - 901
3xFLAG - 943
HindIII - 1007
NotI - 1014
EcoRI - 1021
BglII - 1033
EcoRV - 1041
Acc65I - 1045
KpnI - 1049
XbaI - 1058
Myc - 1063
BamHI - 1097
BbvCI - 1387
BsmBI - 1451
AgeI - 1649
BseRI - 2066
BssHII - 2645
BstBI - 2930
NruI - 3103
AfeI - 3360
MfeI - 3552
SV40 PA - 3555
HpaI - 3565
PciI - 4079
pBR322 ori - 4572
ScaI - 5452
f1 ori
CMV
bGH PA
SV40 ori
NeoR
AmpR
p3xFLAG-CMV25-Myc
6406 bp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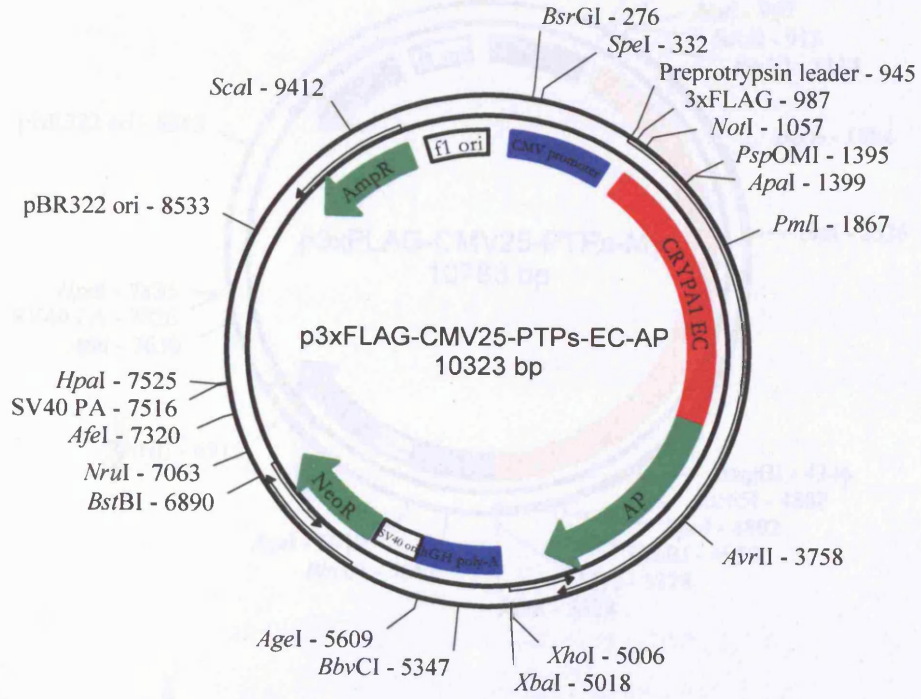
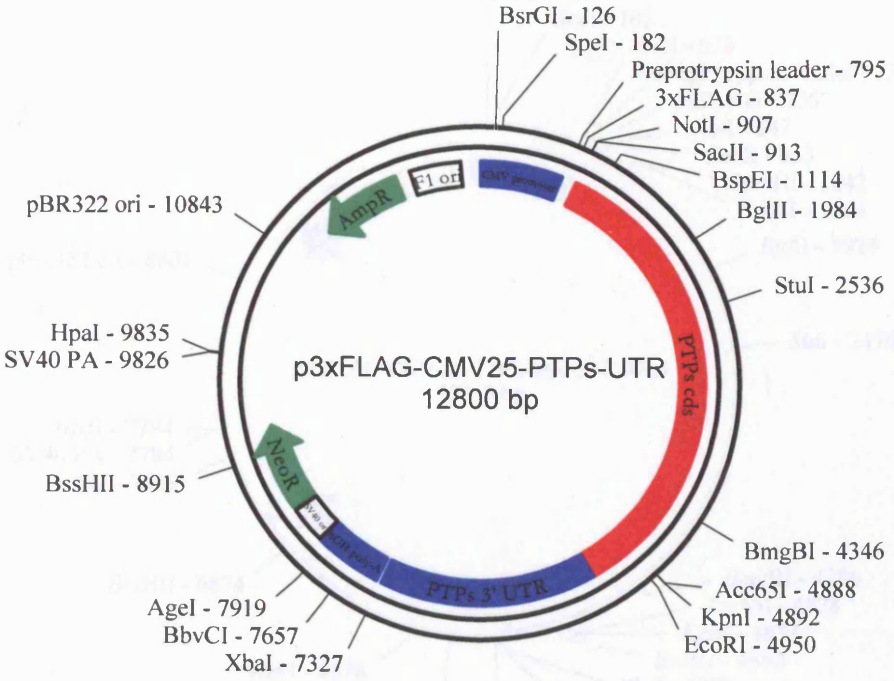
NdeI - 567
SnaBI - 673
Preprotrypsin leader - 945
3xFLAG - 987
NotI - 1057
SacII - 1063
BspEI - 1264
PmlI - 1867
BglII - 2134
StuI - 2686
XbaI - 3552
BbvCI - 3881
AgeI - 4143
BssHII - 5139
NruI - 5597
BstBI - 5424
AfeI - 5854
HpaI - 6059
SV40 PA - 6050
pBR322 ori - 7067
ScaI - 7946
f1 ori
CMV promoter
AmpR
PTPs EC
NeoR
SV40 ori
bGH poly-A
p3xFLAG-CMV25-PTPs-EC
8857 bp


BsrGI - 276
SpeI - 332
Preprotrypsin leader - 945
3xFLAG - 987
NotI - 1057
PspOMI - 1395
ApaI - 1399
PmlI - 1867
AvrII - 3758
XhoI - 5006
XbaI - 5018
BbvCI - 5347
AgeI - 5609
BstBI - 6890
NruI - 7063
AfeI - 7320
SV40 PA - 7516
HpaI - 7525
pBR322 ori - 8533
ScaI - 9412
f1 ori
CMV promoter
AmpR
CRYPA1 EC
AP
NeoR
SV40 ori
bGH poly-A
p3xFLAG-CMV25-PTPs-EC-AP
10323 bp


BsrGI - 126
SpeI - 182
Preprotrypsin leader - 795
3xFLAG - 837
NotI - 907
SacII - 913
BspEI - 1114
BglII - 1984
StuI - 2536
BmgBI - 4346
Acc65I - 4888
KpnI - 4892
EcoRI - 4950
Myc - 5328
XbaI - 5328
AgeI - 5919
BbvCI - 5657
BssHII - 6915
HpaI - 7835
SV40 PA - 7826
AfeI - 7630
pBR322 ori - 8843
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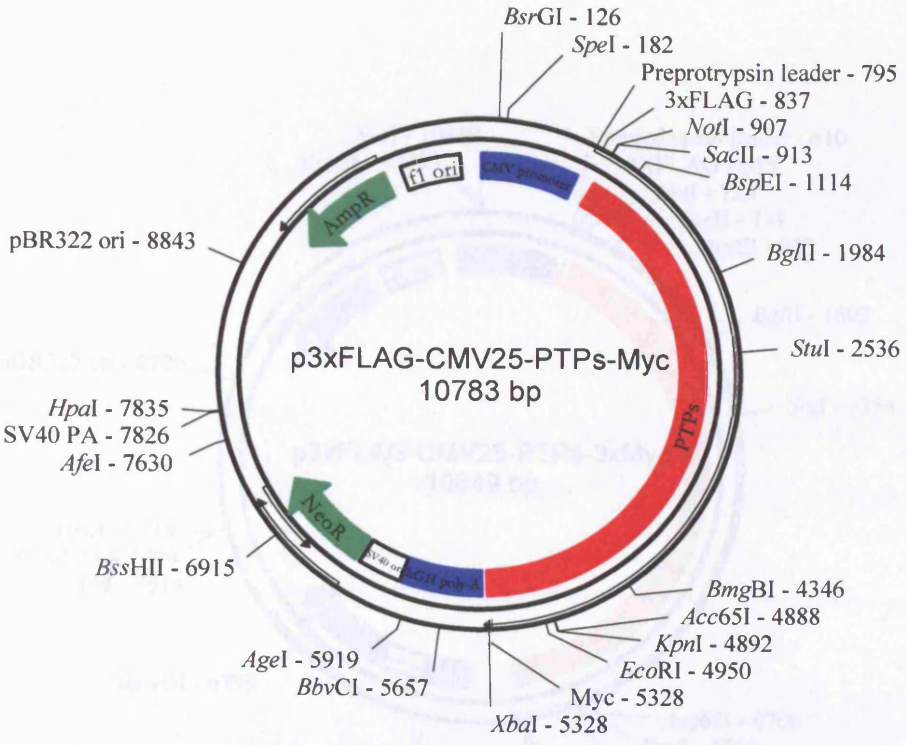
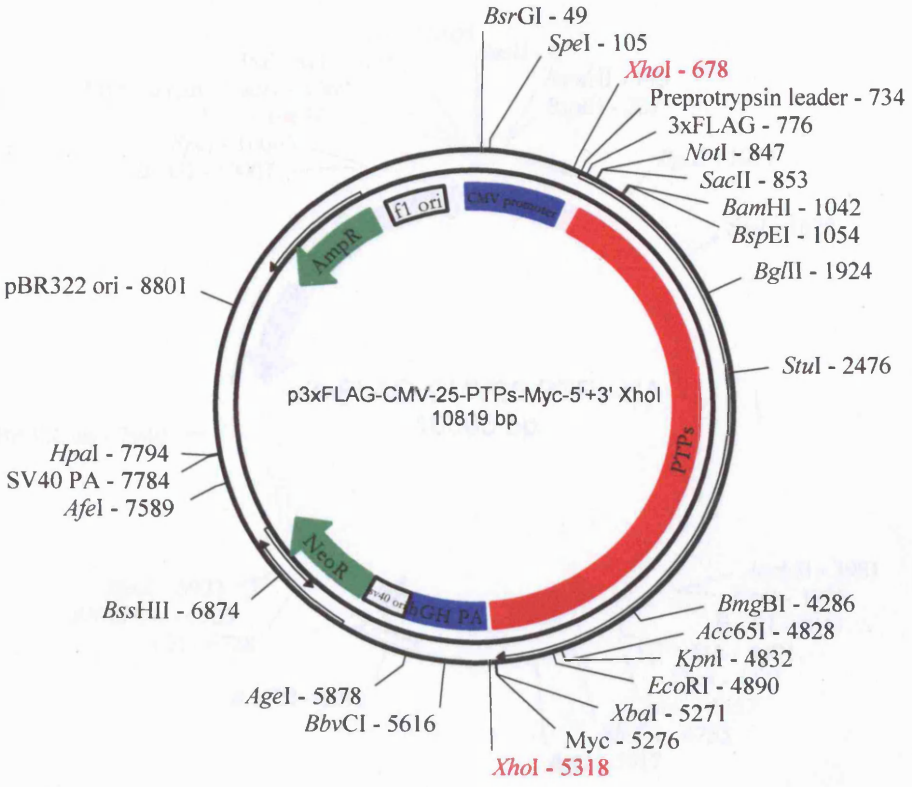
10783 bp
f1 ori
CMV promoter
AmpR
NeoR
PTPs
SV40 ori
SV40 poly-A


BsrGI - 126
SpeI - 182
Preprotrypsin leader - 795
3xFLAG - 837
NotI - 907
SacII - 913
BspEI - 1114
BglII - 1984
StuI - 2536
BmgBI - 4346
Acc65I - 4888
KpnI - 4892
EcoRI - 4950
Myc - 5328
XbaI - 5328
AgeI - 5919
BbvCI - 5657
BssHII - 6915
HpaI - 7835
SV40 PA - 7826
AfeI - 7630
pBR322 ori - 8843
p3xFLAG-CMV25-PTPs-Myc
10783 bp
f1 ori
CMV promoter
AmpR
NeoR
PTPs
SV40 ori
SV40 poly-A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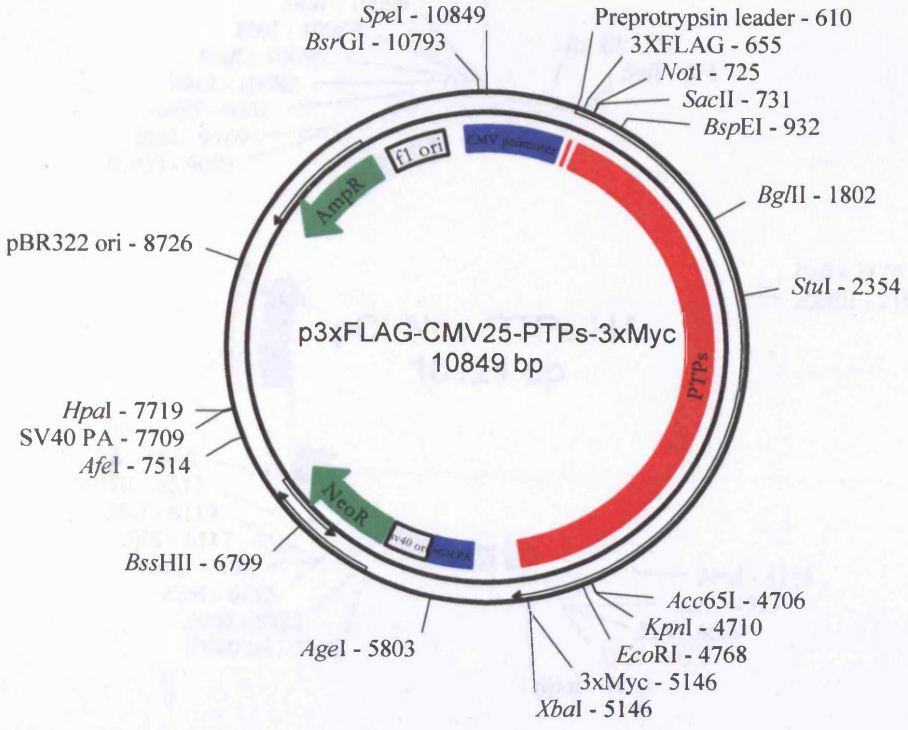
SpeI - 105
XhoI - 678
Preprotrypsin leader - 734
3xFLAG - 776
NotI - 847
SacII - 853
BamHI - 1042
BspEI - 1054
BglII - 1924
StuI - 2476
pBR322 ori - 8801
p3xFLAG-CMV-25-PTPs-Myc-5'+3' XhoI
10819 bp
HpaI - 7794
SV40 PA - 7784
AfeI - 7589
BssHII - 6874
BmgBI - 4286
Acc65I - 4828
KpnI - 4832
EcoRI - 4890
XbaI - 5271
Myc - 5276
AgeI - 5878
BbvCI - 5616
XhoI - 5318
f1 ori
CMV promoter
AmpR
NeoR
bGH PA
PTPs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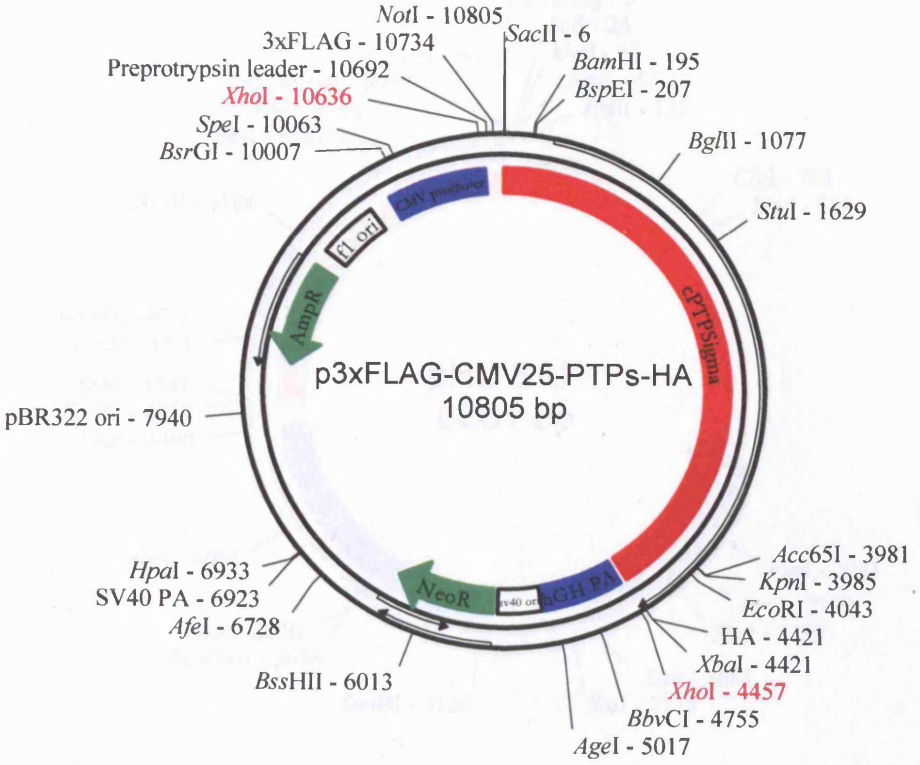
BsrGI - 10793
Preprotrypsin leader - 610
3XFLAG - 655
NotI - 725
SacII - 731
BspEI - 932
BglII - 1802
StuI - 2354
pBR322 ori - 8726
p3xFLAG-CMV25-PTPs-3xMyc
10849 bp
HpaI - 7719
SV40 PA - 7709
AfeI - 7514
BssHII - 6799
Acc65I - 4706
KpnI - 4710
EcoRI - 4768
3xMyc - 5146
XbaI - 5146
AgeI - 5803
f1 ori
CMV promoter
AmpR
NeoR
PTPs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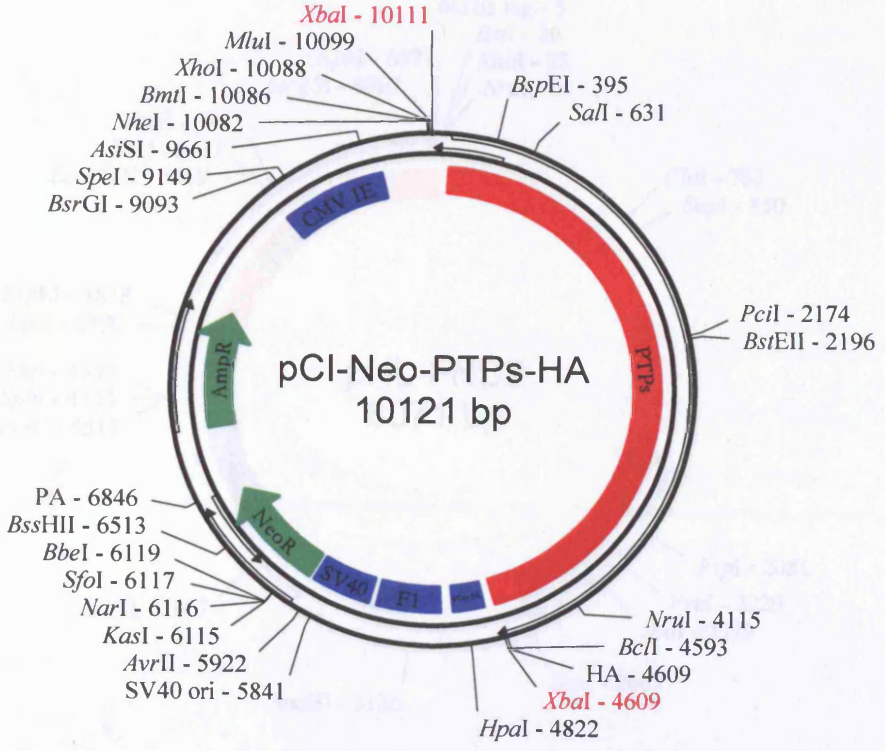
3xFLAG - 10734
SacII - 6
Preprotrypsin leader - 10692
BamHI - 195
XhoI - 10636
BspEI - 207
SpeI - 10063
BsrGI - 10007
BglII - 1077
StuI - 1629
CMV promoter
f1 ori
AmpR
cPTPSigma
p3xFLAG-CMV25-PTPs-HA
10805 bp
pBR322 ori - 7940
Acc65I - 3981
KpnI - 3985
EcoRI - 4043
HA - 4421
XbaI - 4421
XhoI - 4457
BbvCI - 4755
AgeI - 5017
HpaI - 6933
SV40 PA - 6923
AfeI - 6728
NeoR
SV40 ori
BGH PA
BssHII - 6013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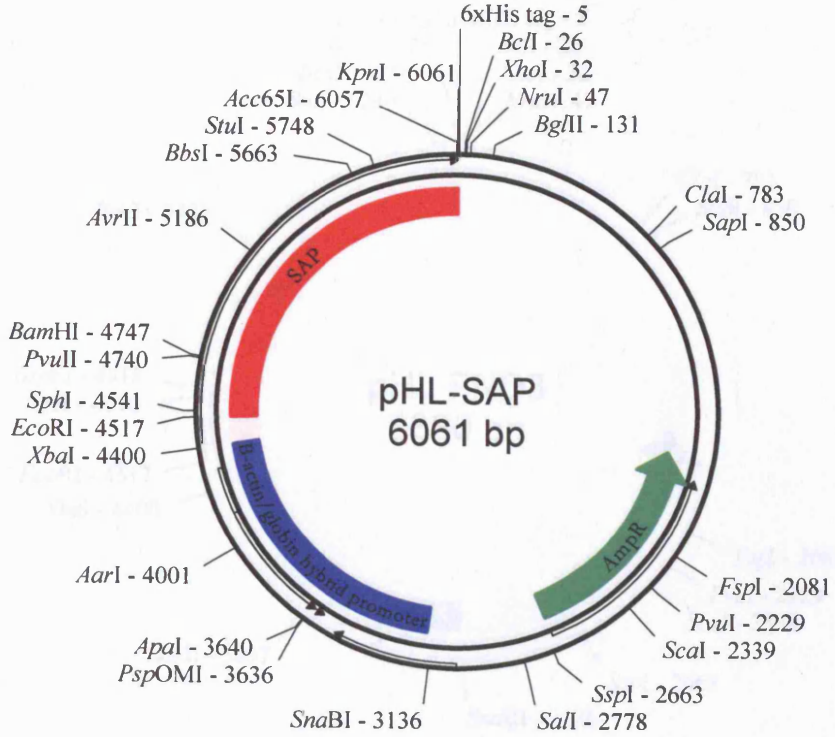
MluI - 10099
XhoI - 10088
BmtI - 10086
NheI - 10082
AsiSI - 9661
SpeI - 9149
BsrGI - 9093
BspEI - 395
SalI - 631
CMV IE
PciI - 2174
BstEII - 2196
AmpR
pCI-Neo-PTPs-HA
10121 bp
PTPs
PA - 6846
BssHII - 6513
BbeI - 6119
SfoI - 6117
NarI - 6116
KasI - 6115
AvrII - 5922
SV40 ori - 5841
NeoR
SV40
F1
NruI - 4115
BclI - 4593
HA - 4609
XbaI - 4609
HpaI - 4822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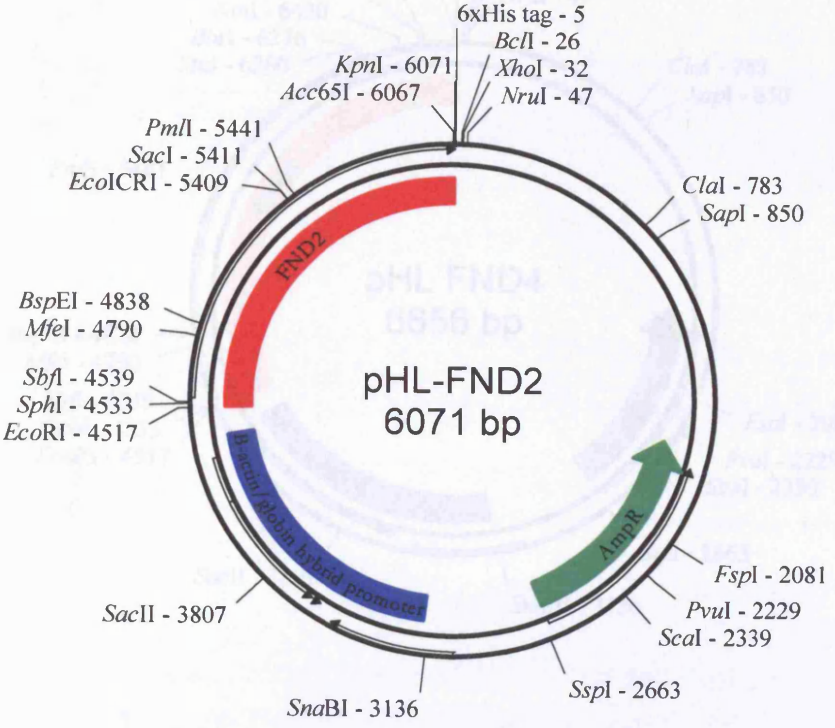
BclI - 26
KpnI - 6061
XhoI - 32
Acc65I - 6057
NruI - 47
StuI - 5748
BglII - 131
BbsI - 5663
ClaI - 783
AvrII - 5186
SapI - 850
SAP
BamHI - 4747
PvuII - 4740
pHL-SAP
6061 bp
SphI - 4541
EcoRI - 4517
XbaI - 4400
β-actin/globin hybrid promoter
AmpR
AarI - 4001
FspI - 2081
PvuI - 2229
ScaI - 2339
ApaI - 3640
PspOMI - 3636
SspI - 2663
SnaBI - 3136
SalI - 2778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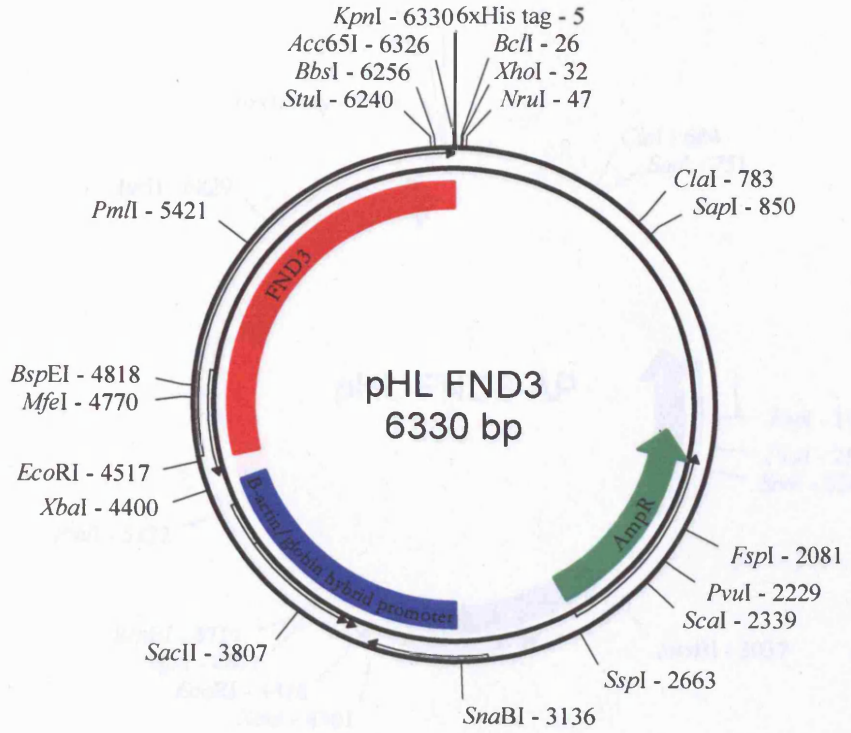
BclI - 26
KpnI - 6071
XhoI - 32
Acc65I - 6067
NruI - 47
PmlI - 5441
SacI - 5411
EcoICRI - 5409
ClaI - 783
SapI - 850
FND2
BspEI - 4838
MfeI - 4790
pHL-FND2
6071 bp
SbfI - 4539
SphI - 4533
EcoRI - 4517
β-actin/globin hybrid promoter
AmpR
FspI - 2081
SacII - 3807
PvuI - 2229
ScaI - 2339
SspI - 2663
SnaBI - 3136


KpnI - 6330
6xHis tag - 5
Acc65I - 6326
BclI - 26
BbsI - 6256
XhoI - 32
StuI - 6240
NruI - 47
ClaI - 783
SapI - 850
PmlI - 5421
FND3
pHL FND3
6330 bp
BspEI - 4818
MfeI - 4770
EcoRI - 4517
XbaI - 4400
AmpR
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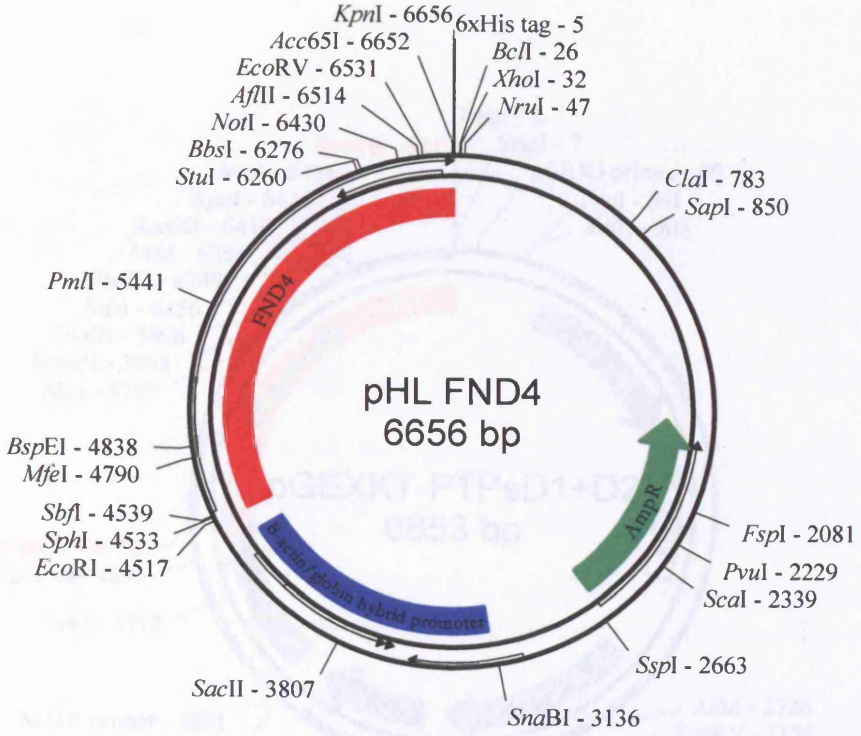
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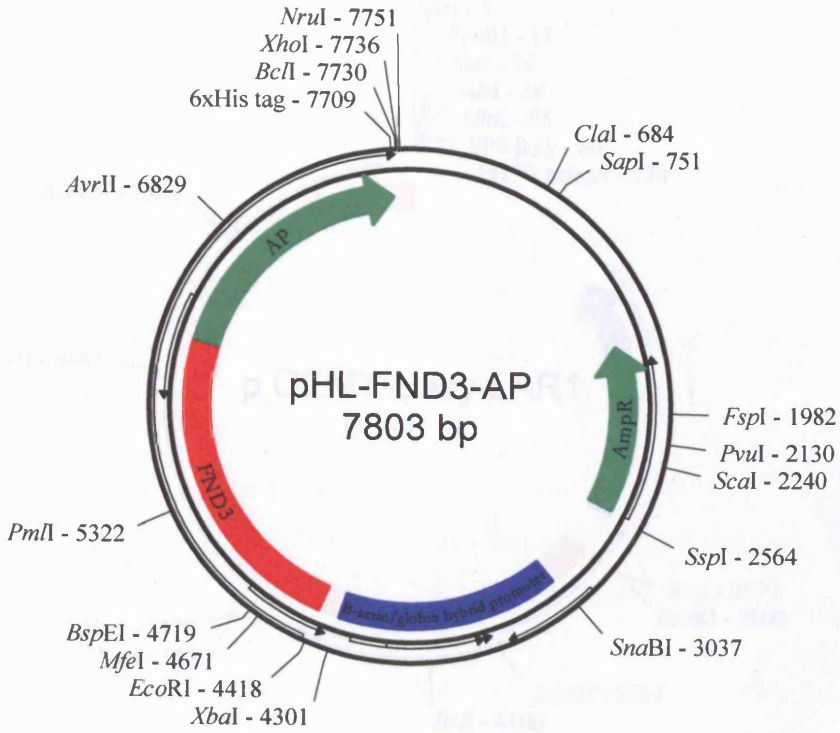
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7803 bp
FspI - 1982
PvuI - 2130
ScaI - 2240
AmpR
FND3
PmlI - 5322
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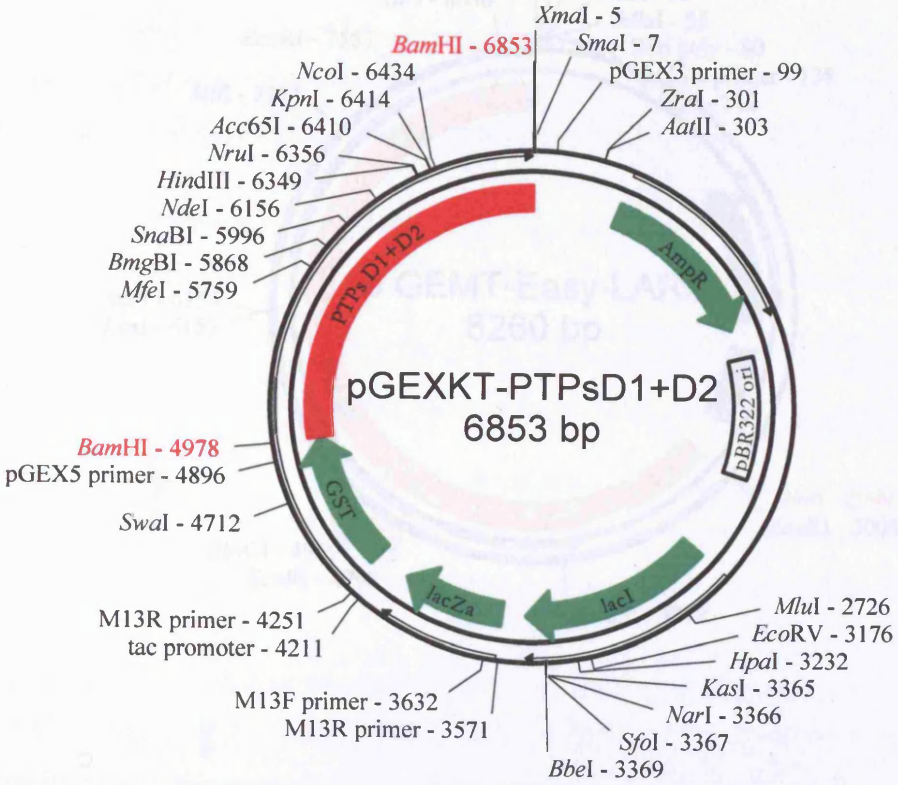
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HindIII - 6349
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SnaBI - 5996
BmgBI - 5868
MfeI - 5759
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AmpR
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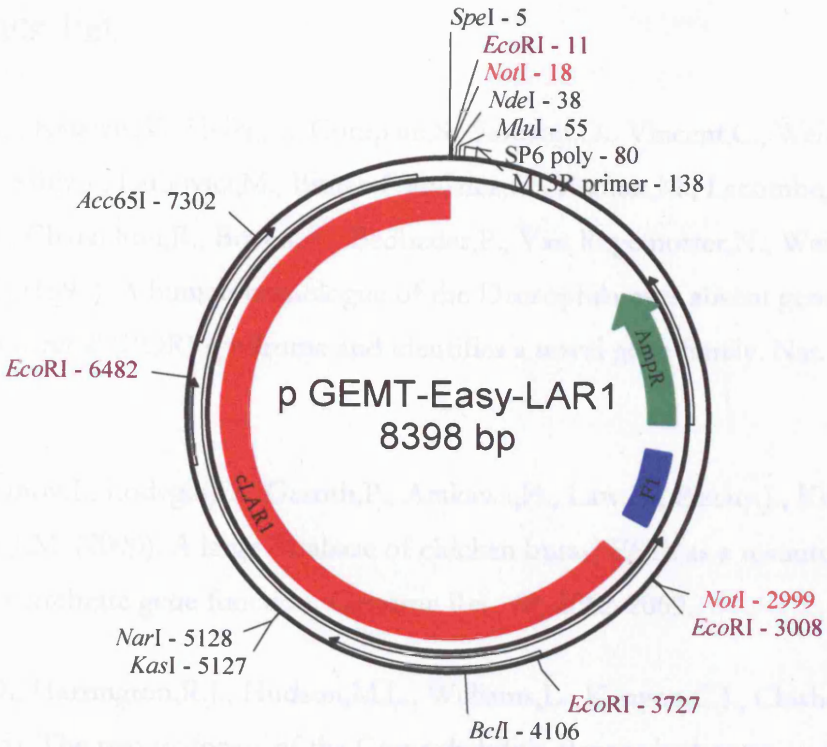
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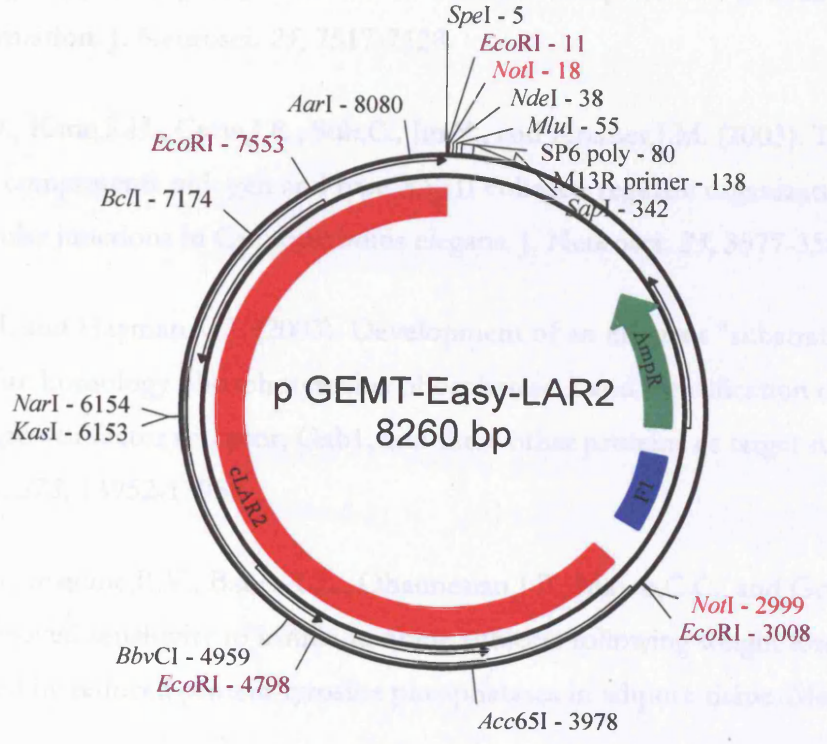
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